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REVIEW ARTICLE **OPEN ACCESS**

Microbial Limit Test [MLT] of Pharmaceutical Product: A Review

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ABSTRACT
Microbiological limit tests are used to measure the presence of certain live bacteria in pharmaceutical medicines or samples, both qualitatively and quantitatively. Pharmaceutical products are closely linked to the development of different dosage forms that are eventually utilized by humans to treat serious illnesses. As a consequence, the test should be done on all dosage forms to guarantee that the formulation is free from contamination and will not harm the human beings as a result of the microorganism. Continuous environmental monitoring samples for microbiological quality of various locations in pharmaceutical facilities are by far the most important measure in controlling clean area and environment, both of which have a serious influence on the microbiological quality of the final pharmaceutical products. Bioburden testing is an essential component of pharmaceutical microbiology since it offers information about pharmaceutical quality during the manufacturing process.Since its first publication in the United States Pharmacopeia (USP) in 1980, the bacterial endotoxin test (BET) has been utilised as a pharmacopeial methodology. This lysate gelation process, known as the Limulus test has been widely used as a simple and sensitive endotoxin detection technique. The most-probable-number (MPN) approach estimates population density without counting individual cells or colonies. It is also known as the method of ultimate or extinction dilution, or simply the dilution method. In a nutshell, the aim is to be able to alter the drug product process to account for variations in Active pharmaceutical ingredient (API) batch key material qualities such that drug product quality remains constant.
Keywords*:Microbial limits; Environmental Monitoring; Bioburden* Testing; Active Pharmaceutical Ingredient; History and Harmonization; Bacterial Endotoxin test.

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INTRODUCTION

Leeuwenhoek, Koch, and Pasteur predicted microbial isolation and identification based on phenotypic investigation of microbial cells by microscopic examination of water, fermented products, and clinical specimens at the dawn of microbiology(1).Microbiological limit tests are used to quantify and subjectively evaluate the amount of particular live microorganisms present in a sample (2). Total aerobic microbial counts (TAMC) and total yeast and mould counts (TYMC) are done on both raw materials and finished products and specific microbiological species are included (*Escherichia coli, Pseudomonas aeruginosa, Salmonella and Staphylococcus aureus*) (3).The microbiological limit test was published by the United States Pharmacopeia [USP] in 1970 based in part on Association of Official Analytical Collaboration (AOAC) International methodology (4). Furthermore, the USP guidelines only apply to specific microorganisms and do not cover all of the FDA's undesirable species. Appendices 16 of the European Pharmacopoeia (EP), the Japanese Pharmacopoeia (JP) and the British Pharmacopoeia (BP)and all provide the microbiological limit test (5).Microbiological limit testing is classified into two categories according to the researchers: Quantitative testing detects the presence of specific pathogen indicators such as *Salmonella spp., Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosaand* the Enterobacteriaceae family which shown in Table.1, while qualitative testing detects the presence of microorganisms such as bacteria, yeast, and mould in a pharmacological specimen.and*E. coli* was identified in the 1940s using a total count on tryptone glucose yeast extract (TGYE) and Eosine methylene blue (EMB) or Endo agar due to its toughness, flexibility, broad palette, and flexibility of handling. It is the most examined and well understood organism on the globe (19) Because hazardous pathogens are

discovered in lower concentrations than non-pathogenic microorganisms, the sample is cultivated in broth for at least 24 hours (20).

In the last 10-15 years, a number of significant natural product-based medical advancements have developed in therapeutically effective medicines in current clinical usage or at various phases of clinical inquiry, mostly from the standpoint of pharmaceutical companies (21). The United States Pharmacopeia (USP) has recently begun demanding bacterial endotoxin criteria based on maximum human dosage for monograph constituents that may be used in sterile products (22).Solid dose forms (capsules or tablets) are frequently spoiled or deteriorated by microorganisms. The most serious issue induced by microbial contamination of solid dosage forms is the absence of visible signs of disintegration (23).

Microbiological testing in clean rooms or controlled environments to observe shifting patterns in microbial counts and microflora growth is referred to as environmental monitoring.The data collected includes information on the physical architecture of the chamber, the functioning of the heating, ventilation, and air-conditioning (HVAC) system, personnel hygiene, gowning processes, apparatus, and cleanliness activities (24).According to the FDA's inspection guidance for equipment cleaning (chemical residues alone), firms must have written protocols (SOPs) defining the cleaning operations, as well as a published general procedure specifying how the cleaning processes will be checked. The FDA is seeking for a final validation report that has been approved by management and states whether or not the cleaning technique is authentic (25).

Environmental monitoring provides the evidence and documentation needed to evaluate the efficacy of various measures for preventing microbial contamination (26). Aqueous and water-soluble products were examined using membrane-filter techniques (27). The direct inoculation approach and tablets were employed to detect *E. coli*: the medium dilution method was utilised to count bacteria, and the usual pour-plate method was used to count fungi and yeasts (28).

History and Harmonization of Microbial Limit Testing

An East Indian Company Dispensary committee lobbied for the creation of a Pharmacopoeia in 1833 and in 1844, the Bengal Pharmacopoeia and Comprehensive Conspectus of Traditional Medicines was produced, which substantially contained the majority of regularly used indigenous medicines (29).Kluyver, Van Niel, and Stainer's research highlighted the importance of microorganisms in substance rotation in the ecosystem, and even the shared biochemical processes of microbes and macroorganisms (30). The purpose of harmonisation is to relieve pharmaceutical manufacturers of the burden of running a test in different methods and using different acceptance criteria to establish that a specific product complies with standards for a certain quality feature (31).

However, the Microbial Limit Testing harmonisation plan will necessitate growth promotion testing with each new lot of media being compared to the previously utilised lot (32). While the International Conference on Harmonisation (ICH) helps the pharmaceutical sector decrease the cost of research and development by avoiding duplication of efforts, it also addresses concerns about wasteful experimentation and the growing expense of healthcare (33). Harmonization challenges began in September 1989, when three pharmacopoeias, USP, JP, and EP, founded the Pharmacopoeial Discussion Group (PDG) to work on excipient harmonisation including standards and test procedures (34). Almost all test technique components, including inoculum size, sampling frequency, recovery medium, neutralising process and result assessment, were harmonised(35). The harmonised preservative efficacy test, particularly the choice of challenge organisms, has a negative impact on many pharmaceutical companies in developing countries; therefore, it is recommended that a statement have been included in the future improvements to allow the use of wild organisms if their expertise for such work is experimentally proven (36).Because of the potential effect on permissible constraints, the necessity to create harmonised monographs may face internal resistance within a single organisation (37).

Microbial Limit Tests

The microbiological limit tests are designed to provide qualitative and quantitative evaluations of certain live microorganisms identified in samples. It includes total viable count (bacteria and fungus) assays as well as an Escherichia coli coliform test. To avoid microbial contamination from the outside, these tests must be conducted with prudence (38). The bioburden test is the 2nd Microbiological Limits test group and it comprises of two independent tests: TAMC detection and TYMC determination (39). When doing a drug microbiological limit test, the technique employed should be validated to ensure that it is appropriate for the drug's microbial limit test as well as the bacteriostatic product because the test bacteria are interfered with during the test settings, the test results cannot accurately represent the number of contaminated microorganisms in the medicine, and specific precautions must be followed. Some designs are not rigorous are not standardized, and cannot accurately represent the test method's integrity and viability (40).

The Most Probable Number Method

It approaches estimates population density without counting individual cells or colonies. It is also known as the ultimate or extinction dilution method or less descriptively, just the dilution method as shown in fig.3(41). The MPN approach is directly applicable in media qualification investigations as well as substitute (quick) microbiological procedures. It has also been proposed as an alternative approach for trend environmental monitoring research (42).A plate count may be readily converted into a number representing the most likely amount of bacteria and this quantity is a constant value of the plate count. With a multiple tube data, however, the most expected number of microorganisms is an exponential proportion of this result, needing further computations to convert the data into the MPN value (43).

Environmental monitoring

It is an essential component of a pharmaceutical manufacturing operation's microbiological quality control system (44). Three problems frequently restrict the usefulness and objectivity of environmental monitoring:

1. Sample count (n), which is frequently constrained by sample analysis and/or collecting expenses.
2.Amount of sample, which is frequently constrained by the procedure utilised.
3. Examine a place, which is frequently restricted by accessibility (45).

Environmental monitoring is often characterised as viable or non-viable. Microbiological environmental monitoring is classified as feasible monitoring and is further segmented into the following sample types: a. settle plates; Volumetric air sampler for active air sampling; b. surface samples: contact plates or swabs; c. passive air sampling; and d. Fingers, sleeves, and other gown regions were sampled by employees (46).

Bioburden Monitoring

The microbial content of a substance (or on its surface) is referred to as 'bioburden' (47). Bioburden monitoring is a regulatory obligation, and the frequency of citations for failing to do so sufficiently is rather high in an examination of Food and Drug Administration warning letters from 2001 to 2011(48). This testing determines the quantities of microbes present in a drug's bulk solution prior to sterilization, providing critical information for the manufacture of a safe product. In general, bioburden testing must be carried out in accordance with the methodologies outlined in the pharmacopoeias (membrane filtration or plate count)(49).

Bacterial Endotoxin test (BET)

It is a straightforward test that has been used as a pharmacopeial method since 1980, when it was originally published in the US Pharmacopeia (USP) (50). Bacterial endotoxin is a lipopolysaccharide (LPS) component of Gram-negative bacteria's cell wall (51).In the current study, the gel-clot, kinetic turbidimetric, and kinetic-colorimetric assay methodologies were used to test for bacterial endotoxin (52).

Maximum Valid Dilution: The LAL test is frequently more sensitive than is required to identify a product's endotoxin limit. As a result, products can be diluted to overcome interference while still permitting detection of the endotoxin limit. By far the most significant approach for managing with interference is dilution. However, a product may not be diluted beyond the point where the endotoxin limit may be detected. This is referred to as the maximum valid dilution (MVD). The MVD is a dilution factor (53).

MVD = [(Potency of Product) × (Endotoxin Limit)] / λ (54).

The MVC of diclofenac sodium was measured for various sensitivity levels. Using the formula MVC = (λ * M)/K, lysate limulus amebocytes where MVC stands for the minimum valid concentration of the tested sample, λ is the sensitivity of the tested sample Limulus amebocyte lysate was employed in tests (in EU/ml) as well as the definitions of M and K characteristics have already been demonstrated (55).

Active Pharmaceutical Ingredient

Before a pharmaceutical industry may get the finished product and tablets, capsules, or other pharmaceutical forms, the following processes must be completed: (a) Active Pharmaceutical Ingredient (API) synthesis, (b) Drug Product (DP) manufacture, and (c) packaging (56).

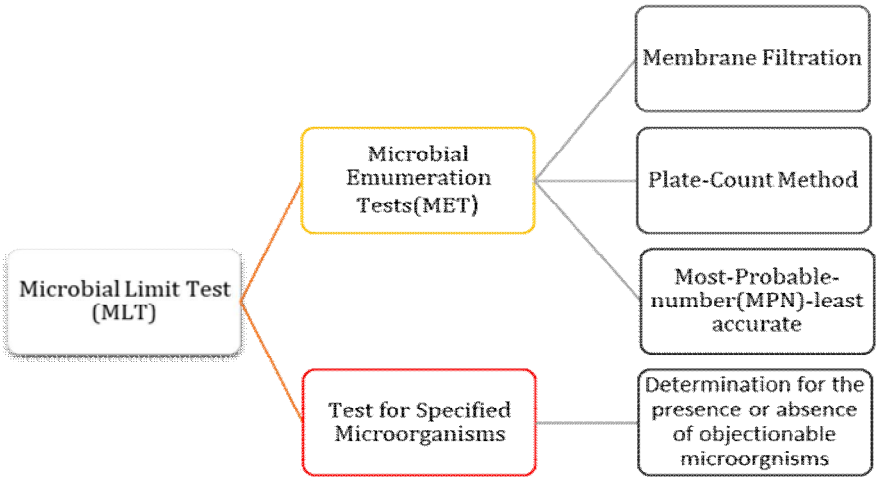

Figure 1. Sub-division of Microbial Limit Test [MLT]

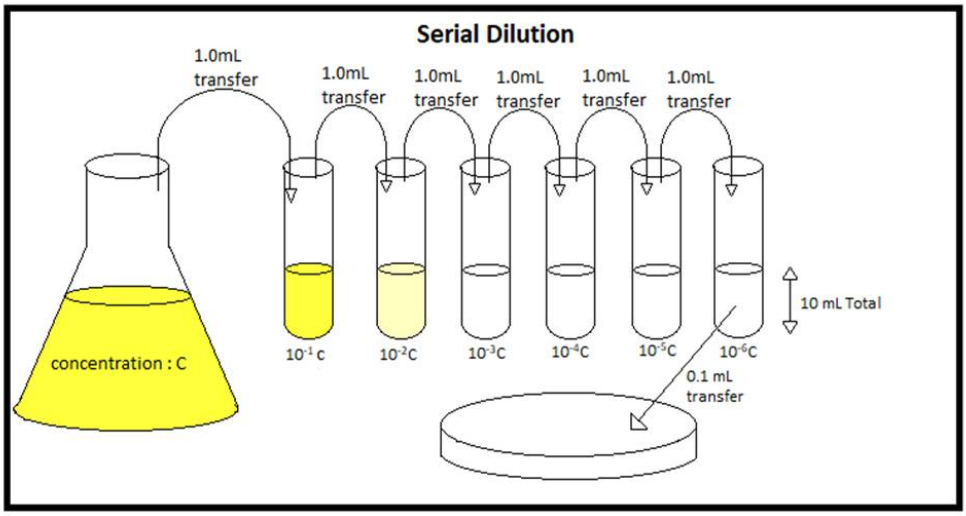

Figure 2. Most Probable Method (Serial Dilution)

Table 1. Typical characteristic of four specified microorganism

SR.no.	Microorganism	Type of bacteria	Shape	Incubation period	Selective media	Culture collection reference	Reference
1	Staphylococcus aureus	Gram positive bacteria (stain purple by gram stain)	Cocci shaped	18º-40º C aerobic or facultative anaerobically	Mannitol Salt Agar	ATCC 6538	6,7,8,9
2	Pseudomonas aeruginosa	Gram negative bacteria (stain pink by gram stain)	Medium rod shaped	37º C for 24 hours	Cetrimide Agar	ATCC 9027	10,11,12,13
3	Escherichia coli	Gram negative bacteria	Bacilli	37º C under aerobic condition	MacConkey Agar	ATCC 8739	14,15,16
4	Salmonella Typhimurium	Gram negative bacteria	Rod-shaped bacterium	37 ºC for 18-24hours	Rappaport-Vassiliadis Soy Broth	ATCC 14028	17,18

CONCLUSION

Microbiological testing for nonsterile medicines is a valid way to assess the risk of significant microbial bioburden and objectionable microorganisms in finished goods and raw materialsbecause a bioburden is authorized in nonsterile pharmaceutical items, the microbiological vulnerability is understood by the type, intended use, and mode of application of the product.Control process and improvement of sterile operations and procedures rely on validation, learning, and documentation of all activities to comply with GMP. Environmental fluctuations are an unavoidable part of every environmental monitoring system. This is because clean chambers and controlled environments are not intended to be sterile, and persistent human and material participation presents a constant challenge to process management and cGMP.

The industries must recognize that worldwide harmonisation of pharmaceutical excipients provides a once-in-a-lifetime opportunity for innovation and the elimination of significant barriers resulting from current inconsistencies, and they must focus attention on and participate with the process.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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