PEER REVIEWED

The Role of the Clean Room in LAL Production

The standard endotoxin test utilises a reagent called Limulus Amoebocyte Lysate (LAL). LAL comes from the amebocytes of the American Horseshoe Crab (*Limulus polyphemus*). It is the proteins that create a coagulation in response to bacterial endotoxin. This test is the most widespread test of bacterial endotoxin in parenteral injections and medical accessories.

In the production of LAL, many invisible factors contribute greatly to the quality of the product. Since it is the *de-facto* standard for the vital detection of endotoxins in pharmaceuticals and medical instruments, the production of the reagents must be at the highest order of cleanliness. The development of clean room technology has created an environment that allows for large scale production of an extremely effective endotoxin test. As clean rooms allowed the LAL test to become a widely produced pyrogen test, clean rooms also allow for the continued development of the next generation of endotoxin tests.

Any material that produces a febrile response is described as pyrogenic. As can be imagined, many materials are pyrogenic. However, one class is especially notorious – bacterial endotoxin. Endotoxins are the lipid A component of the LPS from the outer membrane of gram-negative bacteria. They are the main pyrogen of concern due to their small molecular weight. Most pyrogens are protein complexes and can be eliminated through ultrafiltration or deactivated through denaturisation, but endotoxins cannot be removed through ultrafiltration since they have a molecular weight in the low thousands. They are unaffected at temperatures below the boiling point of water, so steam sterilisation has no effect. The only means to destroy endotoxins is through rapid oxidation at temperatures over 250°C. This is only possible in dry glassware, greatly limiting the ability to remove endotoxins present in aqueous materials or in plastic containers. Protection of pure raw materials is therefore imperative in producing finished products free of endotoxin.

Pasteur's germ theory of disease is the theory behind clean room development and the LAL test. The precursor in practice to the LAL method was the Rabbit Pyrogen Test. This test used the *in-vivo* injection of a sample into a series of rabbits to determine if the sample had any pyrogenic properties. Since the immune response of the rabbit as a mammal is nearly identical to the human response, the lack of a pyrogenic response in the rabbits indicated that the sample would not produce a human febrile response. Of course, the major disadvantage of this test was the need for *in-vivo* tests on living rabbits.

In 1956, the horseshoe crab's clotting response to bacterial endotoxin was first described. In a similar pathway to the mammalian clotting response to interleukins, the horseshoe crab has a clotting pathway directly triggered by bacterial

endotoxins. This led to the first *in vitro* test of bacterial endotoxins using the clotting factors from horseshoe crab hemolymph. This method has become the standard endotoxin testing method, gaining accuracy through quantitative methods. The current developments in endotoxin testing are the manufacture of recombinant factors for an animal-free product and the development of a human monocyte pyrogen test.

The manufacture of production-level LAL reagents faces unique challenges due to the nature of the test. The clotting pathway is a cascade that is initiated by the smallest amount of endotoxin. From the moment the sample is taken from the crab to the final sealed product, the lysate must be kept free of all contamination. Pyrogen-free containers are adequate for protecting small quantities of products. A pyrogen free container can be created as simply as heating glassware. On a small scale, the reagent is kept pyrogen free by always maintaining a sealed barrier. However, in a large-scale production, it is advantageous to not just have the interior of the containers be pyrogen-free, but to extend this level of cleanliness to the environment. This proposal, however, faces several unique challenges. Endotoxin is ubiquitous in the air, particularly in human habitation. As bacterial material, endotoxin levels are not static. Biofilms, colonies, and single bacteria that would test negative will multiply, increasing the level of endotoxins in an area that previously had no detectable levels of endotoxin. Similar problems to these are found in other industries as well such as the manufacture of medical instruments, pharmaceuticals, and the space industry. The solution to these industries including the manufacturing of Lysate is the clean room.

The clean room is designed to produce a space free of airborne particles. Clean rooms are rated by the number of particles in the air, and these different classes are applied to specific needs based on the situation. The heart of the clean room is HEPA filtration. Surprisingly, HEPA filtration was first developed in nuclear science to be able to remove radioactive materials from the air. NASA quickly adopted HEPA filtration and clean room research. Although this may seem surprising, NASA's reason was to prevent biological contamination in space. In fact, the largest ISO 1 clean room is operated by NASA. In subsequent years, the medical and pharmaceutical industries adopted the clean room as well. Clean rooms allow for the mass production of formulations and filling in the LAL industry in a volume that previously would have been unattainable. Instead of needing to limit endotoxin-free substances to the interior of vessels, they can now be transferred and used by machinery in open containers in a clean room without contamination.

Several processes must be in effect for a clean room to successfully be maintained. The clean room is not a static environment – several active processes must be maintained to keep the environment clean. There are four main causes of contamination of a clean room that must be actively removed.

The first is from people. Dead skin and respiration mean that humans are actively producing particles that would contaminate a clean room. Gowning, therefore, is a vital part of maintaining a clean room, something that if neglected will undermine the entire space. This includes creating an impervious barrier between human skin and the environment by utilising commercial PPE such as a Tyvek® suit, goggles, and a mask.

The second cause of contamination is airborne. HEPA filtration alone will not produce a perfectly clean environment. Laminar flow and pressure cascades are what work in conjunction with the HEPA

filters to produce a controlled environment in which the air is replaced regularly. The HEPA output creates high pressure, and the clean room is then designed with progressively lower pressures leading to the exterior. By taking advantage of laminar flow, the entire volume of air from the HEPA filters at the highest pressure cascades downward to each successive chamber. Maintaining laminar flow is vital to the integrity of the clean room. Back currents found in turbulent flow are eliminated, and because of this, many small-scale applications that cannot logistically be performed in a clean room can be performed in a laminar flow hood.

The third cause of clean room contamination is from the materials brought into the space. A clean room may have flawless gowning and air handling. However, this cleanliness is all for nothing if one of the raw materials coming into the manufacturing process is contaminated. There are two aspects of raw material contamination. The first is the danger of raw materials contaminating the final product. Regarding lysate, the raw material is naturally endotoxin free within the biological environment, so the main concern is keeping the lysate in clean containers. Water and other materials added during the refinement and formulation are produced through reconstitution with water from a WFI system and careful testing of all materials coming in for endotoxin. There is also the danger that the materials will contaminate the clean room itself. It is imperative that all volatile liquids including all aqueous materials be kept covered as much as possible to prevent excessive airborne particles from being released. Of course, the purpose of the clean room is to protect the materials inside, but this ensures that any materials entering the clean room will not contaminate other materials already present.

The fourth cause of contamination in the clean room is the growth of microbes. This area is the key portion that must be kept in active maintenance. Microorganisms can go undetected and then become a problem later. Spores, a survival mechanism of certain bacteria and fungi, can survive in hostile conditions while going undetected by testing methods. Another source of microbes that could go undetected is biofilms, especially in the filtration system. Certain bacteria will produce an embedding matrix that will sustain the colony in a state that is extremely hard to detect. No perfectly effective means to remove biofilms exist, so the means to ensure that they do not reach levels that will compromise the cleanliness of the clean room is to actively test to make sure microbial growth is not detected as well as active sterilisation even in clean rooms that do not have any positive microbial tests.

The materials that are permanently in a clean room must be evaluated as well. The furniture in a clean room needs to be carefully selected to be made of materials that will not give off particles. The second material is the flooring and walls. Walls in a clean room will degrade and start giving off particles. Floors in clean rooms will often be sticky to hold particles that land on the floor. The curtains in the chambers will need to be maintained as well. These passive measures will help maintain long-term cleanliness in the clean room.

The clean room provides a remarkable advancement of the twentieth century in sterile manufacturing. What once started with military research and moved over to space manufacturing quickly found valuable application in medical and pharmaceutical manufacturing as well. The specific application that I work with in the production of LAL reagents is perhaps one of the more unusual applications of the clean room. The manufacture of LAL reagents does not have a clear-cut category. They are used and are vital in the production of medical and pharmaceutical supplies and are therefore needed to be subjected to the same levels of cleanliness as materials in those manufacturing facilities. However, the LAL is not a synthetic product. It is of biological origin and therefore the materials cannot be regulated to the extent of most pharmaceutical products. It is this origin that led the FDA to classify and regulate LAL as a biological material rather than a pharmaceutical. The preparation of the lysate for the clean room is a specific process that must keep these features in concern. The first step in lysate production is a completely unsanitary one—the preparation of the crabs. As can be imagined, it would be completely impossible to perform this in a clean room setting. Rather, containers must be brought from a clean setting (often a laminar flow hood) with hypodermic needles that will allow the passage of the hemolymph from the interior of the crab body through a sterile passage into the endotoxin-free container. Through the subsequent steps along the process of lysing the amebocytes to extract and purify the clotting factors, as different materials are added and materials are removed through centrifugation, it is often done in the interior of clean containers. Once the lysate reaches the formulation stage, it can be done in a facility separate from initial lysate extraction in a more controlled environment away from contamination. It is often in this step where the production is moved to a full-sized clean room setting over the laminar flow hoods. This clean room setting is where the formulation, filling, lyophilisation and then sealing of the product takes place.

The clean room has allowed production of the LAL endotoxin test that satisfies the need of the world's pharmaceutical demand. Every pharmaceutical injection along with the accessories and instruments need to be tested for endotoxins. The development of the clean room has enabled this vital production to meet the needs of the global medical and pharmaceutical industry.

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