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INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :9.014(2024); IC VALUE:5.16; ISI VALUE:2.286
UGC Approved (2017), Peer Reviewed and Refereed International Journal
Volume:13, Issue:10(1), October: 2024
Scopus Review ID: A2B96D3ACF3FEA2A
Article Received: Reviewed : Accepted
Publisher: Sucharitha Publication, India
Online Copy of Article Publication Available : www.ijmer.in

DETERMINATION OF MICROBIAL BIOBURDEN AND ENDOTOXINS IN PHARMACEUTICAL WATER

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Abstract:
This study is carried out to produce clean, uncontaminated water as a basic requirement in pharmaceutical industry. The presence of organic micro pollutants in personal care and pharmaceutical products is of great environmental and public health concern. Elimination of hazardous chemicals from water, advanced treatments and processes such as filtration, coagulation etc came into existence to produce highly purified water free from any microbial burden. Purified water is mostly used as an excipient in production of parenteral preparations that are intended to be administered intravenously and should be highly preferred and not contain any fever causing agents like pyrogens which are able to raise thermostatic setting in hypothalamus. Therefore, it is essential to develop sensitive, accurate and rapid methods for its detection. The Rabbit pyrogen test is the first standard technique for endotoxin detection but nowadays, it has been replaced by the Limulus Amoebocyte Lysate test which is most popular for the detection of endotoxins.

Keywords: Bacteriological Analysis, Sputum Sample, Lower Respiratory Tract Infection , East Godavari District , Coastal Andhra Pradesh"

INTRODUCTION: Water is widely used as a raw material, ingredients and solvent in formulation and manufacture of pharmaceutical products, Intermediates, Finished Products and active pharmaceutical ingredients (API). Control of chemical purity and microbial quality in water is important for many of its uses. Water is more complicated than what most people think off. Water is classified into two main categories namely bulk water and packaged water.

Bulk Water:-It means water intended for various uses which is transported from one place to another for human consumption and treatment purposes.

Packaged Water:-It is the drinking water packaged, sterilized to preserve microbial quality throughout their packaged shelf life.

The water which is used for drinking must ensure to be free of any coliform or other potentially pathogenic microbes that are highly vicious and leads to deterioration of the entire formulated product and may be hazardous for the human consumption. To attain the required growth of microbes,disinfectants play a crucial role in controlling the innumerable growth of microbes.

Disinfectants include Chorine, Bromine, Hydrogen peroxide, Formaldehyde etc. These disinfectants react with the organic material found in the water and release disinfectant by-products which include Chloroform, Trihalomethanes,Trichloroacetic acid and chlorite etc.But the extent useof DBPs allow us to consider its affect on water as its overuse may cause damage to the pretreatment operation systems.

The complete removal of these disinfectants may cause compendial growth of microbes again.Therefore limited use of DBPs is used for effective disinfection.When DBP levels get increased,it get minimized by adding disinfectants such as chloramines,ozone and chlorine dioxide.But due to their oxidative properties,they degrade during disinfection and release ammonia which can be pass on to the finished water.Hence the unit operations are designed to remove the disinfectant adequately.

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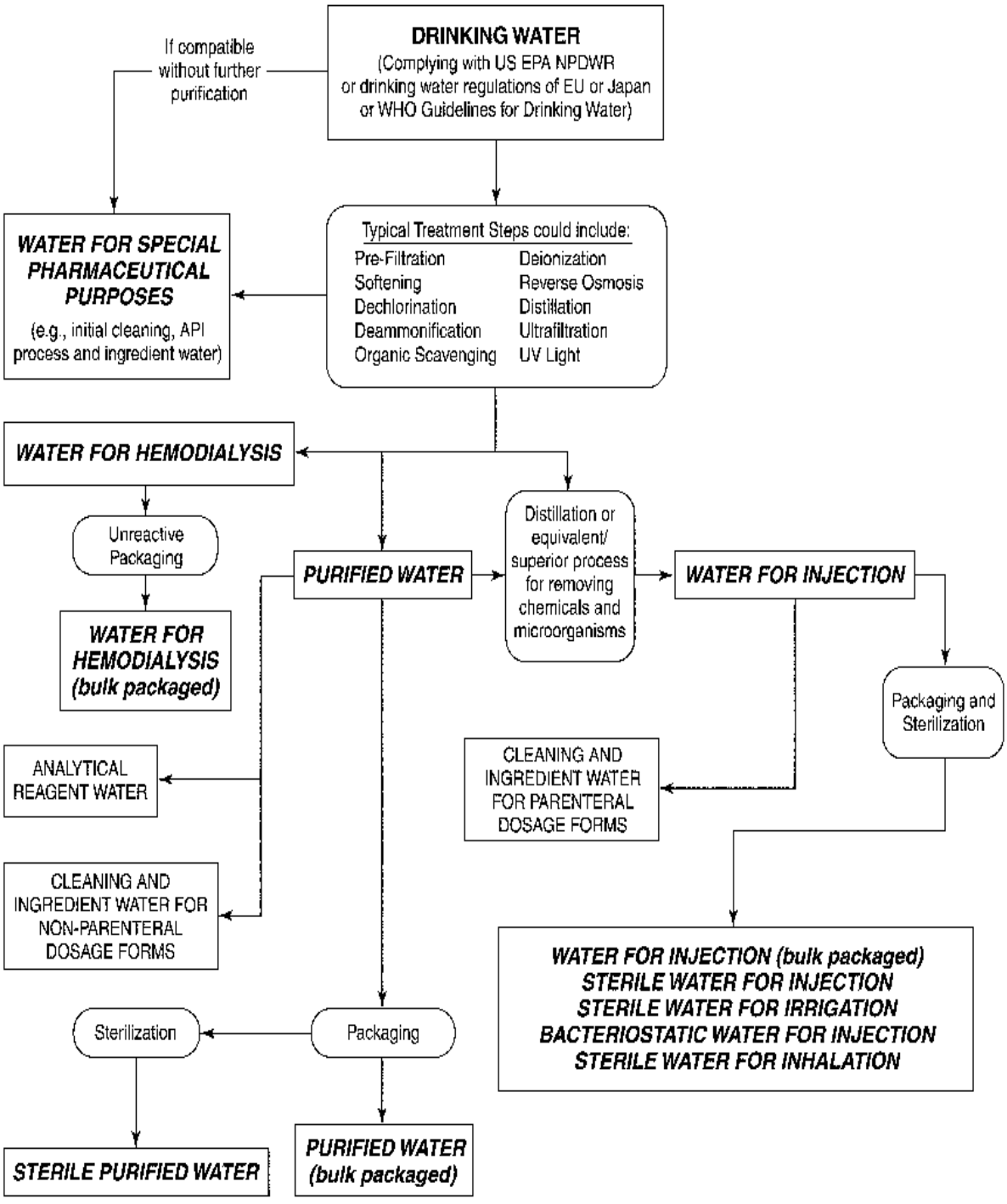

DIFFERENT TYPES OF WATER:

There are different grades of water used in pharmaceutical industry.These water are prepared in bulk volumes by multi-unit operation water system and distributed by piping for use at the same site. These particular water must meet the requirements as needed. These typical waters are classified as follows:

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1. Purified water

2. Potable water.

3. Water for Injection (WFI).

4.Bacteriostatic water for injection.

5. Sterile water for injection (SWFI).

6. Pure Steam Water**.**

7. Water for Hemodialysis.

Purifiedwater:-It is a type of water used in production of non-parenteral preparations and are mostly used for cleaning equipments and it does not contains any contaminants as it is highly purified. It is purified water and must be free of microbial contamination. It must meet the ionic and organic purity and must be free of microbial contamination. The source water is drinking water which is mostly used for the production of purified water which is done by various processes such as deionization, distillation and filtration. It is not used for parenteral preparations.

S.NO	PARAMETERS	LIMITS
1.	APPEARANCE	Clear, Colorless, odorless
2.	PH	Range from 5.0-7.0
3.	CONDUCTIVITY	Not more than 1ms
4.	HEAVY METALS	0.1ppm
5.	TOTAL DISSOLVED SOLIDS	NMT 0.1ppm
6.	MICROBIAL COUNT	Bacteria-100cfu/ml Fungi-10cfu/ml Pathogens -must be absent

POTABLEWATER:-Potable comes from the Latin word ‘potare’ which means “to drink”. It is also called as drinking water which is used mostly for human consumption. It comes from the source and ground water and is treated so that it meets the levels of state and federal standards for consumption. It is not pure water because it always contains dissolved impurities in limited amount. It is mostly produced from sea water by a process called desalination.

S.NO	PARAMETERS	LIMITS
1.	Appearance	Clear, odorless, no visible particles
2.	PH	Between 6.5-8.5
3.	Chloride	NMT 250ppm
4.	Total Hardness	NMT 500ppm
5.	Microbial Count	500 CFU/ml
6.	Specified Pathogens	Must not present.

WATER FOR INJECTION:-WFI is a non-pyrogenic, distilled water used for parenteral preparations. They are sterile and are directly injected into the systemic circulation of the human body. It contains no added substances and should meet

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the requirements of the pyrogen test. Since endotoxins are produced by the kind of organisms that are prone to inhabit water, hence the equipment and systems used should be highly sophisticated and are designed to prevent from microbial growth and free of endotoxins that are coming from the incoming water. It can be stored for periods up to a month in special tanks containing UV lamps.

S.NO	PARAMETERS	LIMITS
1.	Appearance	Clear, odorless
2.	PH	Between 5.0-7.0
3.	Chloride	0ppm
4.	Total Hardness	0ppm
5.	Total Dissolved Solid(TDS)	NMT 10.0ppm
6.	Microbial Count	10CFU/100ml
7.	Conductivity	NMT 0.1ms

BACTERIOSTATICWATERFORINJECTION:-It is a sterile water containing 0.9% of Benzyl alcohol added as a bacteriostatic preservative which is used to dilute or dissolve medications. It belongs to a class of drugs called sterile irrigating solutions. It is only used for dissolving drugs for intravenous, intramuscular injection.It is supplied in a multiple dose container.The semi-rigid vial is fabricated from the polyolefin which is a copolymer of ethylene and propylene. It does not contain any antibacterial agent like other fluids, so that it does not kill the bacteria but it prevent the bacterial growth in water. Usually it does not contain any bacteria but once when used for the first time they get contaminated. This is why the sterile water should be used only once.

S.NO	PARAMETERS	LIMITS
1.	Endotoxin	0.5EU/ml
2.	PH	Between 4.5 and 7.0
3.	Metals	0ppm

SterileWaterforInjection:-It is sterile,hypotonic, non-pyrogenic, distilled water used for intravenous administration after addition of suitable solute. No antimicrobial or other substances are added to the solution. It is used as a sterile diluents for parenteral products. It is not suitable for intravascular administration without being made isotonic by adding solute due to the possibility of hemolysis. The plastic container is made from a multilayer film and do not contains any contaminants and exhibits virtually no leachables. The container is overwrapped to provide protection from external environment and to provide additional moisture barrier when necessary.

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PURESTEAMWATER:-It is used in steam sterilizing porous loads and equipments and in other processes such as cleaning where condensate would directly contact official articles ,containers. Pure steam is also used for air humidification in manufacturing areas where these contact surfaces are exposed to the resulting conditioned air.

The primary cause of using this quality of steam water is to ensure that all articles exposed to it are not contaminated by residues within the steam. Pure steam is prepared from suitably pretreated source water. The contaminants in pure steam should be from source water droplets, steam additives, from distribution systems. These attributes are measured on the condensate and imparts great cleanliness to PSG because it must not adversely impact quality of resulting condensating fluid.

- Analysis of pure steam:-

1.Non –condensable gases:-These are air and CO_2 those do not condense with the steam. These are generated due to their presence in purified water systems and it should be less than 3.5%.

2. Steam dryness value: - Dry steam has more energy than wet steam as it contains water and dry steam has heat energy. Dryness of steam should not be less than 90%.

S.NO	PARAMETERS	DESCRIPTION
1.	PH	Between 5-7
2.	Conductivity	NMT 1.3muS/cm
3.	Endotoxins	NMT 0.25EU/ml
4.	Microbial Count	Must not be present.

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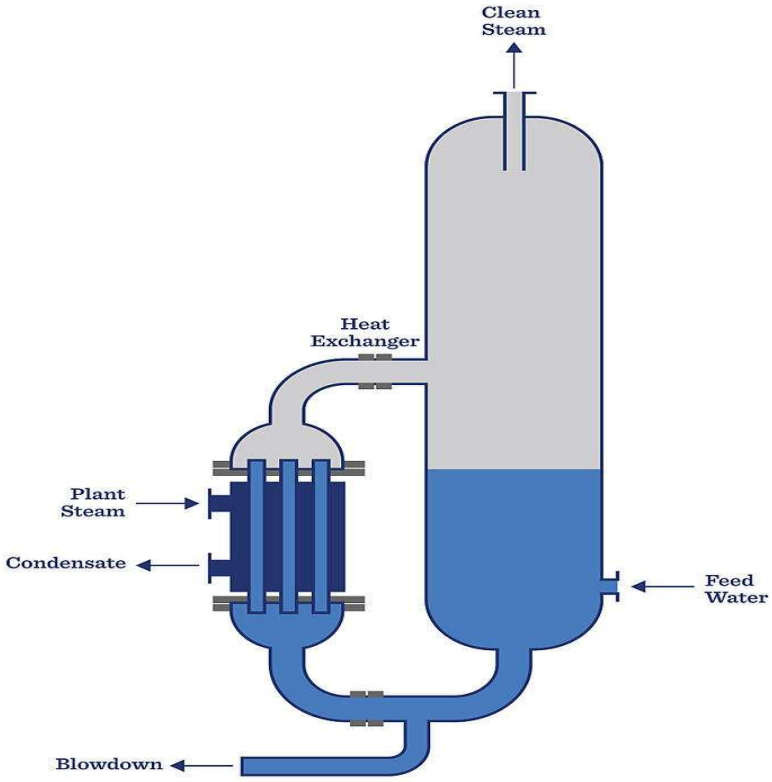

WATER FOR HAEMODIALYSIS: Chemical and microbial contaminants that can be found in drinking water meets US (EPA) may have the potential to produce significant negative affects in patients undergoing hemodialysis. Hence it is necessary to subject the water to further treatment to reduce components to acceptable levels. Excess levels of aluminum, Fluorides and chlorides may be found in drinking water as a result of chemicals used in water treatment system. These components should be monitored in Water for Hemodialysis being produced in accordance with standard operating procedures.

S.NO	PARAMETERS	DESCRIPTION
1.	Calcium	2(0.1mEq/ml)
2.	Mercury	0.0002
3.	Lead	0.005
4.	Microbial limit	100 CFU/ml
5.	Endotoxins	2 EU/ml
6.	Arsenic	0.005
7.	Free Chlorine	0.50

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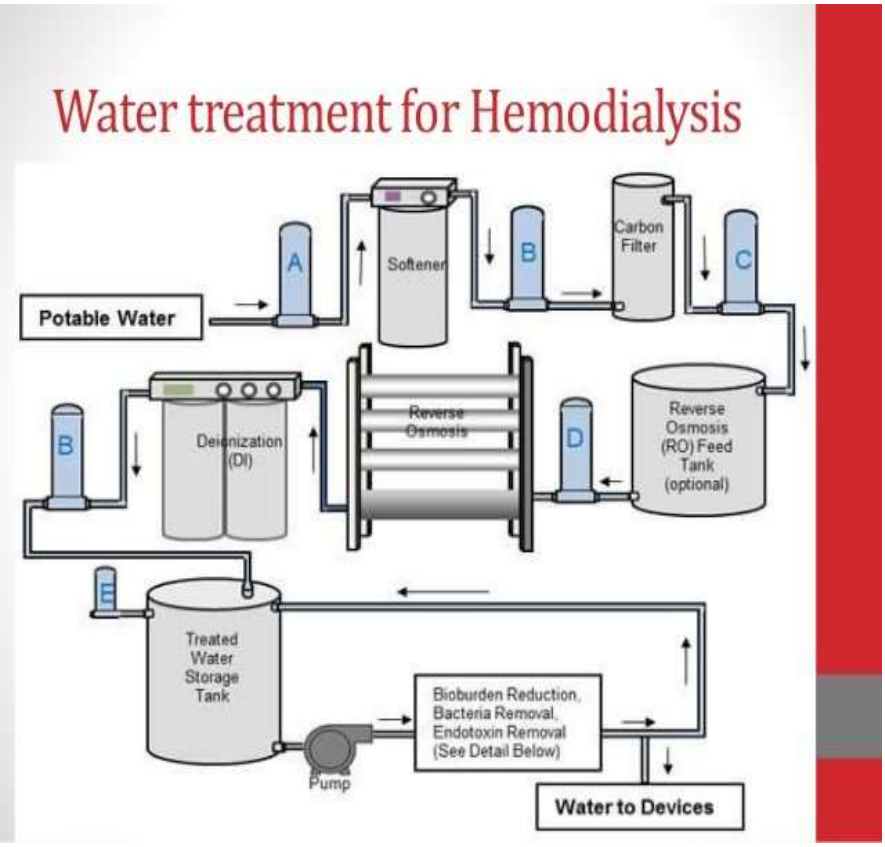

"WATER TREATMENT METHODS"

It is a process involving different types of operations (physical, chemical and biological) and the main aim is to eliminate or reduce the contamination and to eradicate the non-desirable characteristics of water. Waste water discharge from industries, agriculture pollution, environmental and global changes is the main sources of water contamination.

Even traces levels of heavy metals, dyes, and microbes are hazardous to the human health, aquatic system and environment. Water treatment is increasingly necessary due to drinking water shortages. Of the planet's total water reserves,only 2.5% is freshwater and of this amount only 0.4% is fit for human consumption. There are different types of methods used in water treatment. They are given as follows:-

- **Chlorination**:

  Generally, the source of raw water is stored in a storage tank for several days and they are not cleaned for many days which enhance the growth of microbes and leads to contamination of the whole water system. It is a common problem in pharmaceutical industry and can be solved by chlorination process.

  Sodium Hypochlorite is used as a foremost source for the chlorination in water treatment. When NaClO is dissolved in water it gives free chlorine and produce hypochlorous acid which is mostly used in disinfection process.

Cl_2+H_2O ----------->HOCl+ HCL.

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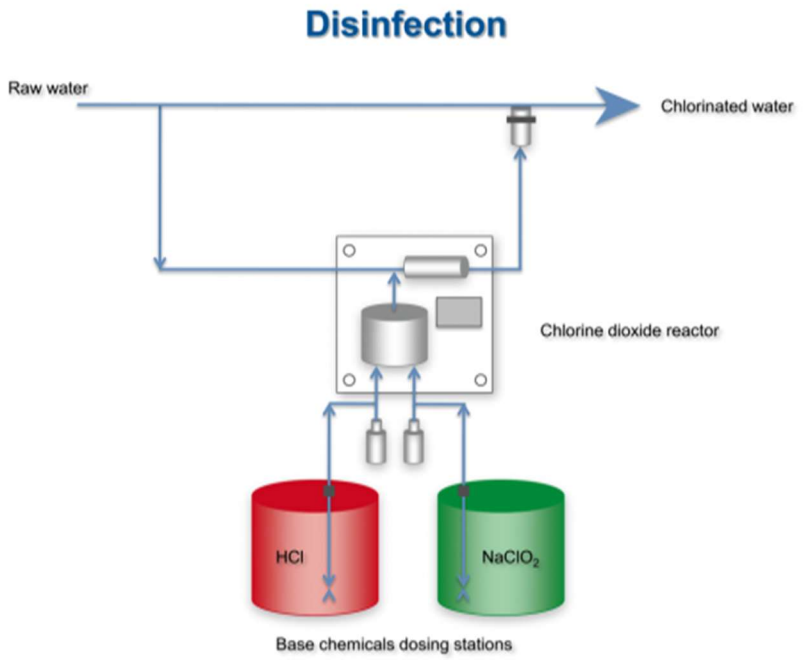
Hypochlorous acid is a neutrally charged molecule which easily penetrates into the cell wall and reacts with lipids results in inactivation of their functions. A 5ppm concentration of chlorine in water is effective. It takes approximately 30 minutes to kill all the pathogens in the water.



- **FILTRATION:**In Raw water pretreatment portions the water is filtered through two filters namely:
- Multi-grade filter
- Activated Carbon filters.

Multi Grade Filters: The Multi Grade Filter(MGF) is generally used for the pretreatment in the water purified system. MGF removes the solid suspended particles such as dust and dirt presentin water. It has a longer life and requires low maintenance costs.

In this the raw water flows into filter inlet through media bed. The media bed comprises different layers such as pebbles, sand etc. As the water passes through various media layers, filtration takes place and it retains all the dust and water flows out of the filter outlet.

Activated Carbon Filters: It is the process which is used toremove organic compounds and chlorine from water. These filters are based on principle of adsorption i.e, it treats and absorbs impurities on the surface.

It is used to removecontaminants such as organic material and remove odour and often used to make drinking water more palatable. Activated carbon is highly porous. Adsorption is directly related to surface of area of media.

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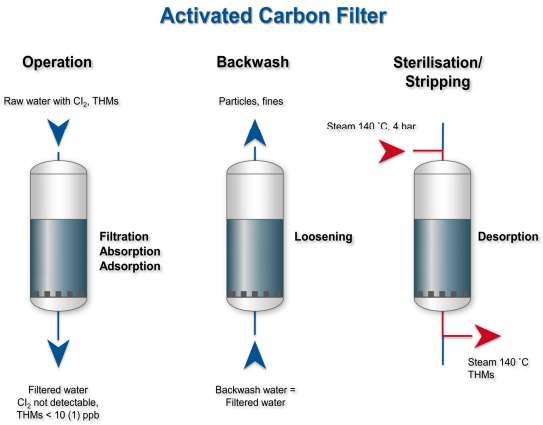


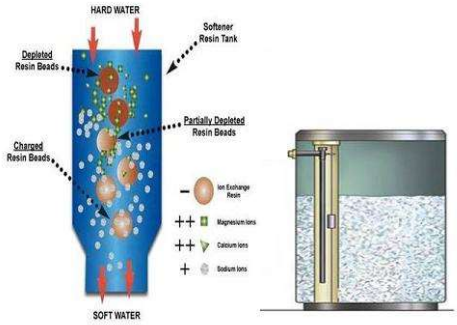
- **SOFTENING:** Pharmaceutical water usually contains a lot of suspended material as well as in the form of ions. Turbidity and suspended solids are removed by activated carbon filters but ions cannot be removed by ACF.

  It is also called as Hardness of Water. Hardness is denoted by Calcium and Magnesium ions. The process of removing this hardness is called Softening. Generally, ion exchange resins are used for the process. These ions are taken by the resin. The resins are charged by chemicals to release this hardness.

ION EXCHANGE RESINS: These are white to yellow colored synthetic polymers beads of small size ranging from 0.5-1.0mm having pores on their surface to trap and release the ions easily. Two types of resins are used and are as follows:

- **CationicExchange Resin:** These resins release hydrogen ions in water while trapping the ions from the water and are negatively charged resins. The resin undergoes regeneration by 8-12% HCL and pH of the water released from the bed is 2-3.
- **Anionic Exchange Resin:** These are positively charged resins and release OH ions in exchange of hardness ions such as magnesium. Thus sodium ions are released in water and calcium ions are attached with the resins. The resins undergo regeneration by treating it with 10-15% NaOH solution. By this process sodium ions are attached with surface of ion exchange resin to get replaced by calcium and magnesium ions of hard water.

Water Softener



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- **ULTRAFILTRATION:** "Ultra filtration" is a process of separation whereby a solution containing a solute of molecular dimensions significantly greater than that of the solvent is depleted of solute by being forced under a hydraulic pressure gradient to flow through a suitable membrane.
  It is used to describe separations involving solutes of molecular dimensions greater than ten solvent molecular diameters, and below the limit of resolution of the optical microscope.Ultrafiltration is usually the method of choice from the point of view of speed, efficiency, and cost, particularly when thermally unstable or biologically active materials are involved, or when large volumes of dilute solutions are to be processed. The UF membrane is a super fine filter that reduces particles 5,000 times smaller than human hair.
  An ultra filtration filter has a pore size around 0.01 micron and remove some viruses also. Other type of filters are Ceramic Ultra filters that are used to remove high molecular weight suspended solids which remain in retentate and low molecular weight solutes pass through membrane in permeate (filtrate). The pore size is 0.01-0.05 micrometer.

ANTISCALING: Antiscalant are pre treatment balls/chemicals that are used in Reverse osmosis water purification process to prevent the Reverse osmosis membrane from scaling. These are also known as scale inhibitor that are used to minimize the potential of the scale that deposits on the surface of a RO Membrane and reduces its flow rate.

Scale formation on the surface of membrane can be prevented by adding antiscalant to the feed water.It is widely used to avoid scaling by salts like silica, iron, barium sulphate, calcium carbonate, gypsum etc.

MATERIAL AND METHOD :

TEST - I: MICROBIAL LIMIT TEST

It is also called as Bio burden test which is widely used to assess the viable micro-organisms that are present in non-sterile pharmaceutical products, health care and cosmetics that can range from raw material to finished products for quality control purposes. This test is designed to determine the presence or absence of microbes in a product.

Prior to performing MLT testing on a product, the method must be validated to ensure that the product has no microbial inhibitory properties which could result in false negatives. It is also known as MLT Method Suitability Test. If these antimicrobial properties are present, these can be eliminated by dilution, filtration, neutralization or inactivation before testing the product. Microbial Limit Test is classified into two types and are as follows:

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1. QUANTITATIVE METHOD.

2. QUALITATIVE METHOD.

1. QUANTITATIVE METHOD: It is mostly used for the quantitative enumeration of only mesophilic bacteria and fungi that are present in the given sample which grows under aerobic conditions. Hence these are also called as Total Aerobic Microbial Count (TAMC) and Total Combined Yeast and Mould Count (TYMC). As it is quantitative method, the results are given in the form of number of colonies present in the given sample per ml i.e. CFU/ml.

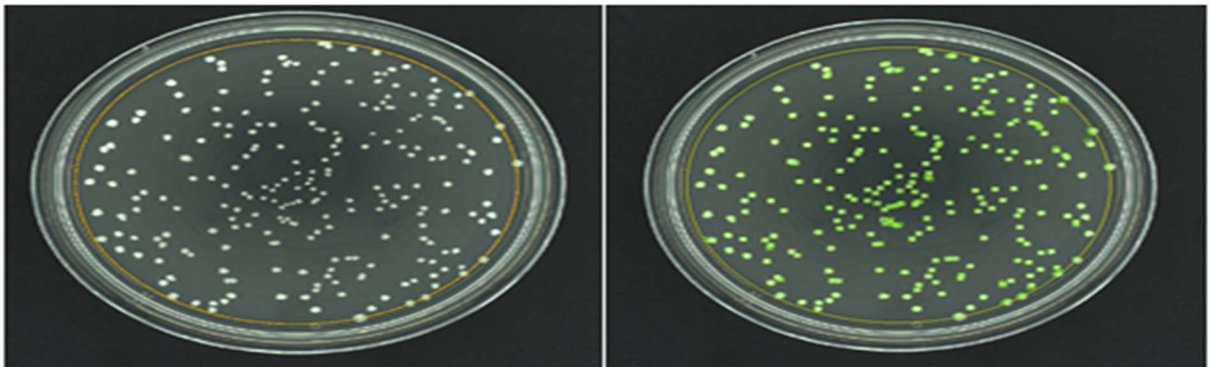
COLLECTION OF SAMPLES FROM SAMPLING POINTS AND ITS SAFETY MEASURES:

Every analyst should carry sample containers to the sampling points in a stainless steel sampling kit. Before going to sampling point he/she must have to carry water sample label book, Technical information (TI) sheet, 70% IPA,stop watch and sterile gloves. Sampling person should follow man and material movement for entry to sampling areas and should follow appropriate GLP's and wipe hands before and after sample collection. If the hose pipe is attached to the point then water should be drawn from the end of the hose pipe and it shouldn't be removed. Clean exterior surface of sampling point with 70%IPA and should not touch the opening point which may cause cross contamination. Water to be tested must be flushed for 30 seconds by recording time. Water flushing moves water systematically creates a scouring action to clean the line. The increased flow rate scours the water pipe's inner walls and helps to remove build-up of naturally occurring debris and sediment. Transfer the sample containers in to SS sampling kit and are transferred into the cooling chamber within 2 hours of collection. The water sample must be tested within 10hrs after collection. Water which is used in pharmaceutical industries are potable water, purified water, WFI, the microbial contamination and the estimation of total number of colonies in the given sample is done by two methods:

A.POUR PLATE METHOD.

B. MEMBRANE FILTRATION METHOD.

A.POUR PLATE METHOD:-This technique is used to perform viable plate counts, in which the total number of colony-forming units within the agar and on the surface of the agar is enumerated. Viable Plate count helps to standardize and calculate concentration of cells on a plate and to evaluate the various environments or growth conditions. It is used to detect lower concentrations on the agar plate due to large sample volume. It requires no pre-drying of the agar.



MATERIALS REQUIRED:-

Procedure For Total Aerobic Microbial Count (TAMC):- Transfer aseptically 1ml of water sample into each of the sterile 90mm Petri dishes. Add previously boiled 20-25ml of R2A agar medium on to the petridish cooled at about 45 C. Cover the petri dish and mix the sample with the agar by rotating the petri dish in both clockwise and anti clock –wise

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direction. Allow the agar to solidify. After solidification, invert the petri dish carefully and incubate them at 30-35 C for 5 days.After incubation observe the plates for any growth detection and if, count the number of colonies per ml.Carry a negative control test for the media by adding 15-20ml of R2A agar medium in a sterile petri dish. The microbial limit for Potable Water TAMC =500CFU/ml.

❖ **OBSERVATION:-**

S.NO	SAMPLING POINTS	TYPE OF SAMPLE	CONTROL	PLATE 1
1.	V201.1.5	Potable Water	Positive Control	**10**

S.NO	SAMPLING POINTS	TYPE OF SAMPLE	CONTROL	PLATE 2
1.	V201.1.24	Potable Water	Positive Control	**15**

S.NO	TYPE OF MEDIA	CONTROL	PLATE 3	CFU/ml
1.	R2A media	Negative Control	Nil	Nil

CALCULATION:

$$= \frac{\text{Colonies on plate 1} + \text{Colonies on plate 2}}{2} \times 100$$

$$= \frac{10+15}{2} \times 10$$

= 12.5 X 10 = 125CFU/ml.

RESULTS: The above value indicates that the water sample contains microbial colonies within the specification limits as per USP 61 and USP 62.

Procedure For Total Combined Yeast and Mold Count (TYMC):- Transfer aseptically 1ml of water sample into each of the sterile 90mm Petri dishes. Add previously boiled 15-20ml of Sabouraud's Dextrose Agar medium on to the petri dish cooled at about 45 C. Cover the petri dish and mix the sample with the agar by rotating the petri dish in both clockwise and anti clock –wise direction. Allow the agar to solidify. After solidification, invert the petri dish carefully and incubate them at 20-25 C for 5 days. After incubation observe the plates for any growth detection and if, count the number of colonies per ml. Carry out a negative control test for the media by adding 15-20ml of Sabouraud's Dextrose Agar medium in a sterile petri dish. The microbial limit for Potable Water TYMC =10CFU/ml.

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OBSERVATION: -

S.NO	SAMPLING POINT	TYPE OF SAMPLE	CONTROL	PLATE 1
1.	V201.1.5	Potable Water	Positive Control	Nil

S.NO	SAMPLING POINT	TYPE OF SAMPLE	CONTROL	PLATE 2
1.	V201.1.24	Potable Water	Positive Control	Nil

S.NO	SAMPLING POINT	TYPE OF MEDIA	CONTROL	PLATE 3
1.	**-**	Sabouraud's agar	Negative Control	Nil

RESULTS: Based on the above observations the given water sample do not contains any fungal or mould colonies. Hence colony formed per ml is to be "Nil".

COLONY COUNTER:-Taking a total microbial count is regular procedure in food, diary and pharmaceutical industry. Manual methods for enumerating bacterial colonies labor-intensive and tiresome process. Colony Counter provides an alternative solution to this problem by helping in fast and accurate counting of colonies It is an instrument used to count colonies of bacteria or other microbes on agar plate. It works by placing a petriplate on electronic pressure pad with light illumination and marking each colony by touching plate with a felt tip pen. The pressure of the touch registers a count in the digital display.

b. Cellulose Acetate Membrane Filters:-These are made of mixtures of cellulose diacetate and triacetate and are hydrophilic in nature. These are prepared by PEG, cellulose acetate and acetone. The pore size of filter completely depends upon polyethylene glycol (PEG).

These filters can be sterilized by autoclaving for microbiological analysis and are biodegradable. These are used in RO and ultra filtration membrane.

Materials Required:-.

Procedure: Aseptically connect the rubber tube of sterile manifold to receiver tank and receiver tank rubber pipe to vacuum pump.Use membrane filter having a nominal pore size not greater than 0.45mu meter. Aseptically transfer 20ml of the water sample onto the filter cup and filter the water sample by applying vacuum. With the help of sterilized forceps, transfer the filtered membrane on to surface of sterile R_2A agar plate. Incubate the plates at temperature of 30-35 C for 24 hrs. Transfer aseptically 10ml of the sterile phosphate buffer solution or BSCP or sterile milli Q water, filter it and transfer the filter paper on to the surface of agar plate to serve as negative control for both media and diluent. Incubate the plates at 30-35C for 24hrs

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Article Received: Reviewed : Accepted
Publisher: Sucharitha Publication, India
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OBSERVATION:

S.NO	SAMPLING POINTS	TYPE OF SAMPLE	CONTROL	CFU
1.	PWPOU-24/K	Purified Water	Positive Control	**60**
2.	**-**	BSCP/Phosphate Buffer	Negative Control	**Nil**

CALCULATION:

Number of colony produced/20mlof water sample = 60.

Dilution Factor = 10.

CFU/ml = Number of colonies produced / Dilution Factor.

= 60/10 = 6CFU/ml.

RESULTS: The result obtained is 6 CFU/ml and it can be written as <01CFU/ml.

S.NO	TESTS PERFORMED	SPECIFICATION LIMITS
1.	POTABLE WATER TAMC	500CFU/ml
2.	POTABLE WATER TYMC	10 CFU/ml
3.	PURIFIED WATER TAMC	100 CFU/ml

2. QUALITATIVE METHOD: These are the tests which are mainly performed to know if the given sample contains a specific organism or not. In these tests the results are not given in the form of counting or calculations. It includes test for specified pathogens.

PROCEDURE TO OBSERVE SPECIFIED PATHOGENS IN THE GIVEN SAMPLE: Transfer aseptically 100 ml of given water sample on to the filtration funnel and allow it to filter completely. After completion of filtration, transfer the membrane filter in to 100ml bottle containing enrichment broth like Soybean Caesin Digest Medium (SCDB) with the help of a sterile forceps. Incubate the broth for 24hrs at 30-35C and allow it for turbid formation. Negative controls are placed by adding sterile purified water sample such as Milli-Q water and continue the process.

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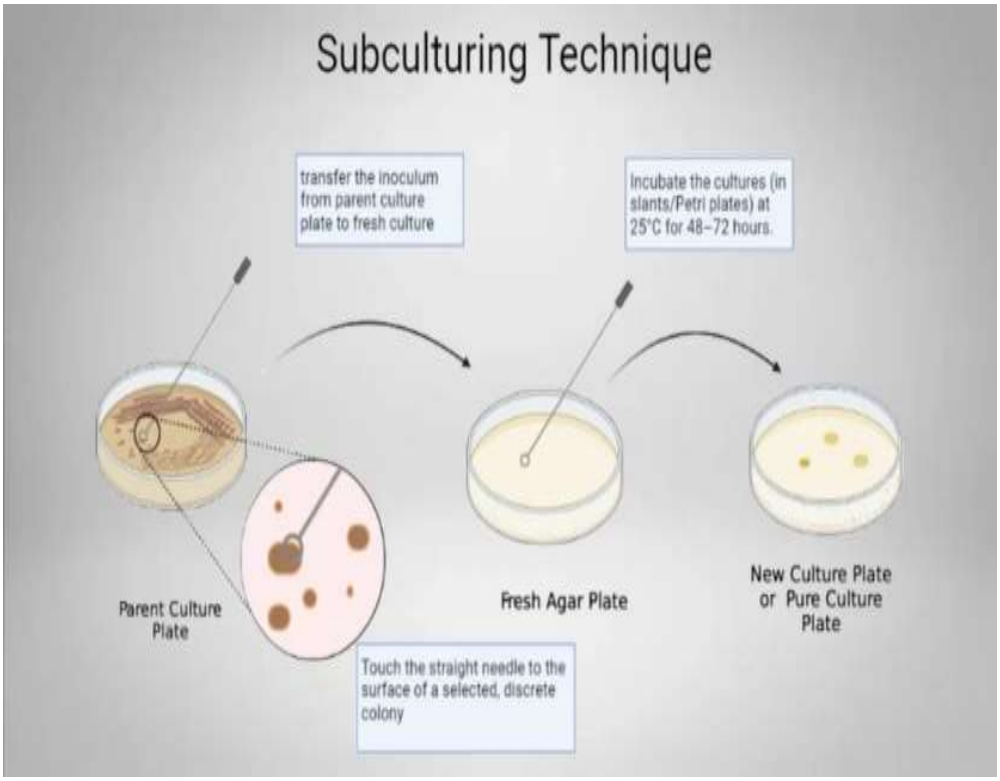
SUB-CULTURING: Sub-culturing is a procedure of transferring of microorganism into fresh nutritive medium from its stock culture. It includes transfer of culture from slant to slant, slant to plate, plate to plate, solid medium to broth and vice-versa. Sub-culturing is done to maintain culture in its active form (prolonging life and/or increase the number of cells) for various applications**.**



ESCHERISCHIA COLI: E. coli is a gram negative, facultative anaerobe, non-sporulating, coliform bacteria. It can live on a variety of substrates and undergo mixed acid fermentation in anaerobic conditions. It is a versatile host for the production of heterologous proteins.

IDENTIFICATION OF E.COLI: Transfer 1ml loop full of suspension from Soybean Casein Digest Broth (SCDB) into 100 ml of Macconkey Broth and mix gently. Incubate the media for 24-48hrs at 42-44 C.After incubation, sub culture the sample by taking loop full of Macconkey broth and allow to streak on Macconkey agar plate (quadrant streak) and incubate the plates for 48hrs at 30-35 C. Carry out the negative control by incubating the media simultaneously along with positive control plates without any inoculation of the culture. Observe the growth of red colour colonies surrounded by precipitated bile on the plate after incubation.

OBSERVATION:-No colony characteristic growth was observed in the plates.

RESULT: The above test indicates theabsence of E. coli in the given water sample.

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BIOCHEMICAL TESTS: These are the tests which shorten the time required to identify microbes, reduce cost and ensure the accuracy of identification of an unknown sample. It is the fastest developing trend in microbial identification.

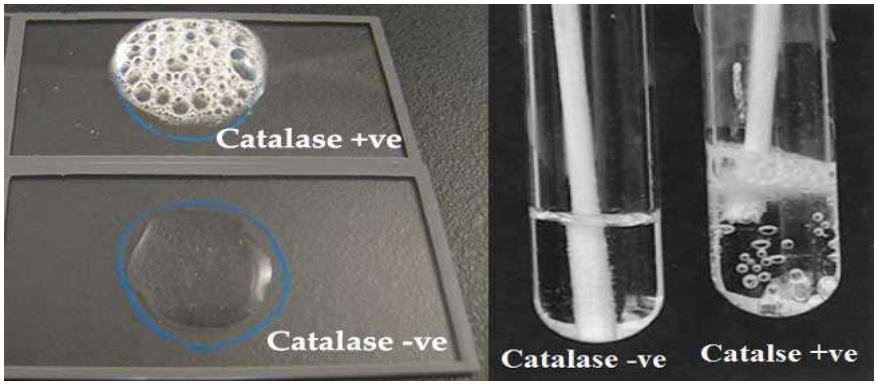
These are used for the identification of bacterial species based on the differences in the biochemical activities of different bacteria. Some of the biochemical tests are given as follows to identify the presence of E. coli in the given sample:-

CATALASETEST: Catalase is an enzyme produced by microbes which live in oxygenated environment to neutralize toxic forms of oxygen metabolites like H_2O_2.Catalase enzyme neutralize the bactericidal affects of hydrogen peroxide and protects aerobes.

PROCEDURE: Transfer a small amount of bacterial colony from subculture to surface of a clean, dry glass slide/a tube using a loop. Place a drop of 3% H_2O_2 onto the slide and mix. A positive result is the rapid evolution of oxygen and evidenced as formation of bubbling.

Catalase +ve: Effervescence (bubble formation).

Catalase -ve: NoEffervescence (bubble formation).



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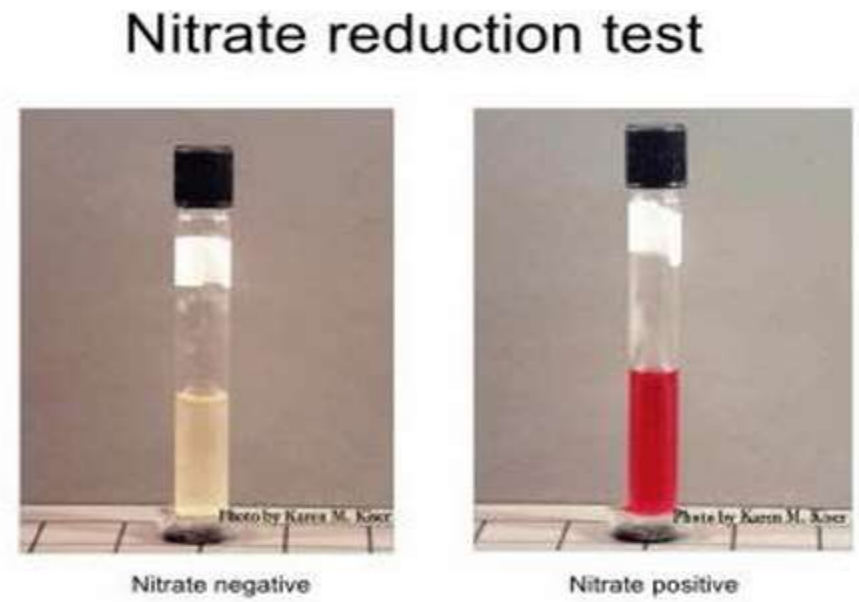
Result: The above test confirms if the given sample contains aerobic bacteria like E.coli.

NITRATE REDUCTION: Many gram-negative bacteria use nitrate as the final acceptor. It involves the production of an enzyme called Nitrate reductase which results in reduction of nitrate.

PROCEDURE: Inoculate the broths with bacterial suspensions.Incubate the tubes at temperature of 30-37 C for 24rs.Add 6-8 drops of Nitrite reagent A and B Observe for the color formation. If no colour observed, add zinc powder.

OBSERVATION: Appearance of red colour in the test tube.

RESULT: The above test confirms if the given sample contains E. coli.



IMViC TEST:IMViC reactions are a set of four useful reactions that are commonly employed in the identification of members of family enterobacteriaceae. They are designed to differentiate Gram – negative intestinal bacilli of family enterobacteriaceae which contains a large number of genera that are biochemically and genetically related to one another. IMViC tests consist of four different tests each of the letters in "IMViC" stands for: I - Indole M - Methyl red V - Voges- proskauerC – Citrate.

INDOLE TEST: Many bacteria use amino acid tryptophan as a source and produce Indole with the help of tryptophanase.

Tryptophan---------- Indole + Pyruvic acid + H2O.

PROCEDURE: Bacterium which is to be tested is inoculated in peptone water. Incubated the solution overnight at 37 C. After incubation, add 4-5 drops of Kovac's reagent on to the test tube. Leave for 2-3 minutes so that reagent comes to the top of the test tube.

OBSERVATION: Red color ring observed on top of the test tube.

RESULT :The above test confirms if the given sample contains E.coli.

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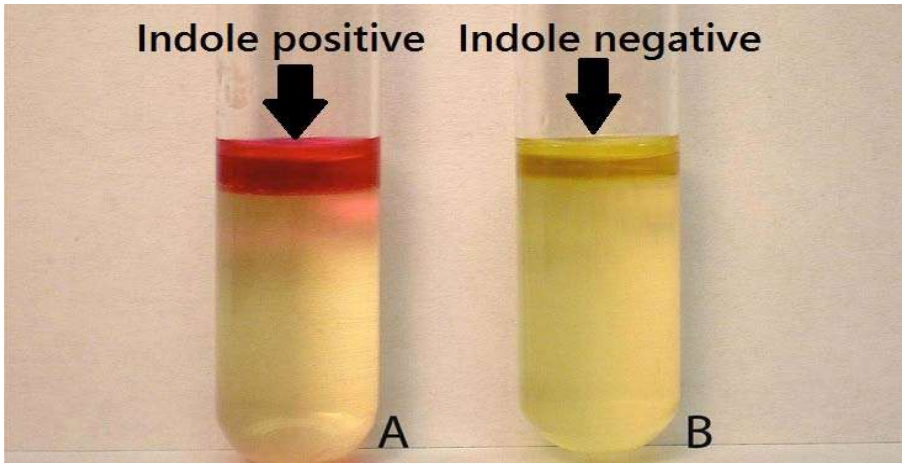
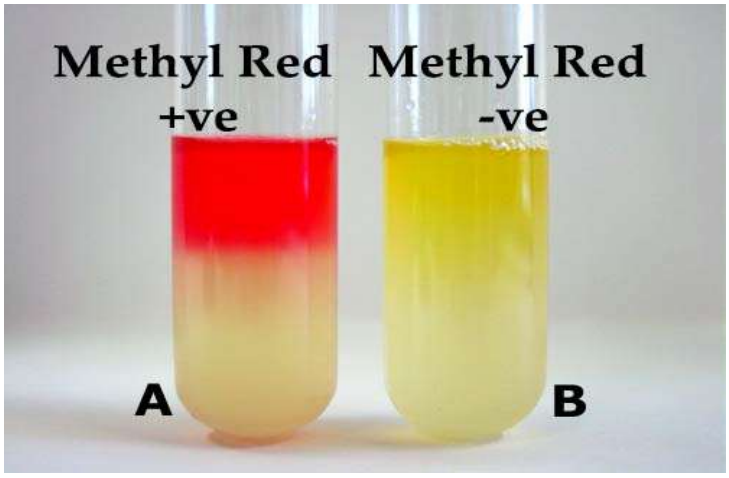


METHYL RED TEST (MR): Organisms have an ability to produce a stable acid after glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation so that they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4.

PROCEDURE: The bacteria to be tested is inoculated in Glucose Phosphate Broth and incubated at 37 C for 48hrs. Over the 48 hours the mixed-acid producing organism must produce sufficient acid to overcome the phosphate buffer and remain acid. The pH of the medium is tested by the addition of 5 drops of MR reagent.

OBSERVATION: No color change observed in the above test tube.

RESULT: The above test confirms the absence of E. coli in the medium.



VOGES-PROSKAUER TEST: VP test detects butylene glycol producers. Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol. In this test two reagents, KOH and alpha-naphthol are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and

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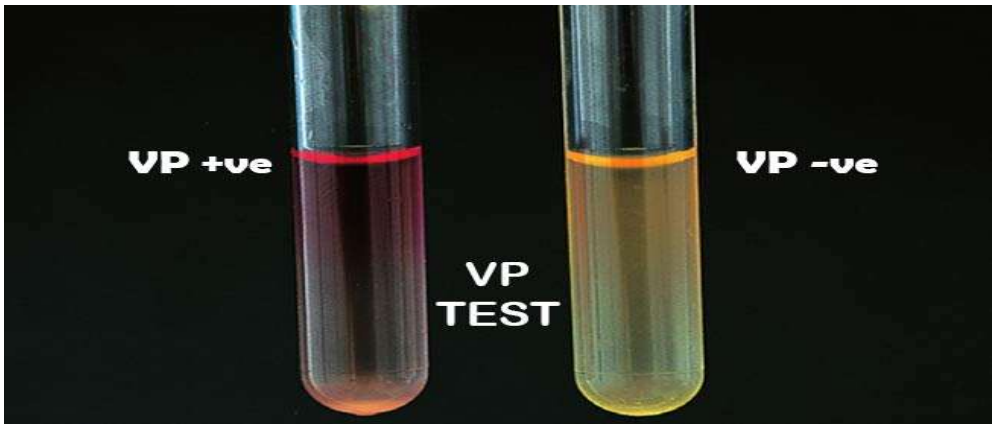
KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alpha- naphthol to produce red colour.

PROCEDURE: Bacterium to be tested is inoculated into glucose phosphate broth and incubated for at least 48 hours. Later add 0.6 ml of alpha-naphthol and 0.2 ml of 40% KOH is added to the broth and shaken. The tube is allowed to stand for 15 minutes.

OBSERVATION: No appearance of red color in the test tube.

RESULT: The above test confirms if the given sample contains E.coli.



CITRATE UTILIZATION TEST: This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Utilization of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate.

Oxaloacetate is further broken down to pyruvate and CO2. Production of Na2CO3 as well as NH3 from utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in change of medium's color from green to blue.

Citrate----------> Oxaloacetate + Acetate.
Oxaloacetate----------> Pyruvate + CO_2.
CO_2+Na+ H_2O----------> $Na_2C_2O_3$.

PROCEDURE: Bacterial colonies are inoculated on slant of Simmon's citrate agar and incubated overnight at 37C. If the organism has the ability to utilize citrate, the medium changes its colour from green to blue.

OBSERVATION: No colour change occurs in the above test.

RESULT: The above test confirms if the given sample contains E.coli.

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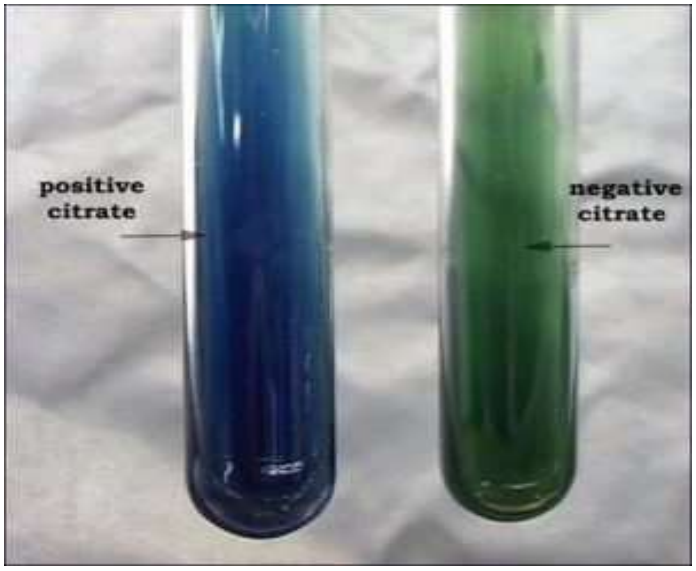
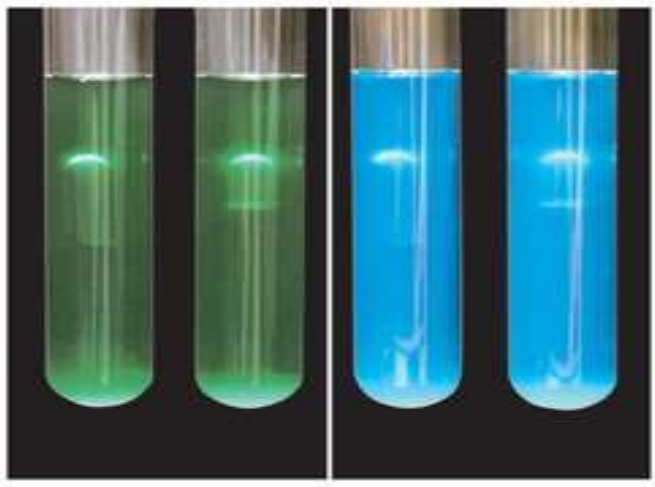


BRILLIANT GREEN BILE LACTOSE BROTH TEST: It is formulated for presumptive identification and confirmation of coliform bacteria. Ox bile and brilliant green inhibit the growth of gram positive bacteria such as clostridia. Production of gas from lactose fermentation is detected by incorporating inverted Durham's tube, indicates a positive evidence of faecal coliforms. Other non-coliform bacteria also grow in this medium but do not produce any gas. It is mostly used in examination of water samples.

PROCEDURE: Dissolve approximately 40g of the powder in distilled water.Distribute to convenient flasks or pour 10ml into each test tubes containing inverted Durham's tubes.Sterilize it by autoclaving at 121 C for 15 minutes. Inoculate 1ml of sample to the test tube and incubate at 44C for about 48hrs.

OBSERVATION: The medium becomes yellowish green in colour and gas production observed in durham's tube.

RESULT: The above test confirms if the given sample contains gram negative bacteria like E.coli.



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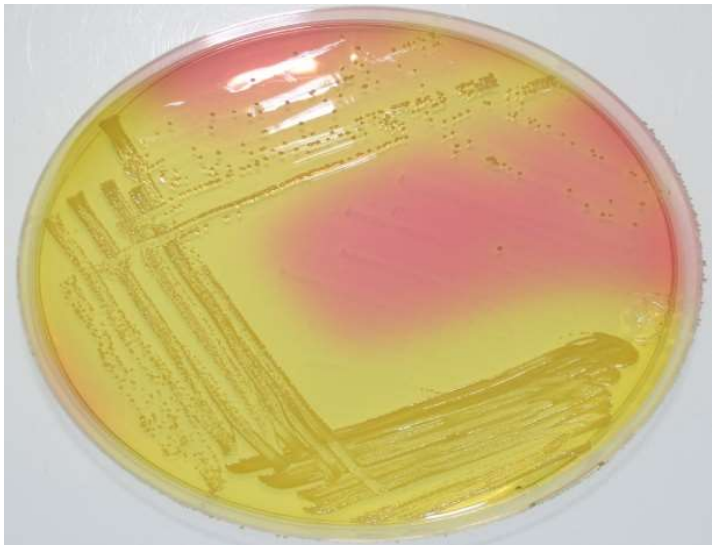
STAPHYLOCOCCUS AUREUS: It is a gram positive, round shaped bacterium found in upper respiratory tract. It is a common opportunistic pathogen and cause food poisoning and sinusitis in humans. It is also called "golden staph". It is non-motile and does not form spores.

IDENTIFICATION OF STAPH: Streak 1ml of enrichment broth onto the Mannitol Salt Agar and maintain as a positive control. Maintain a negative control for the media without inoculating any bacteria. Invert the plates and incubate at 30-35C for 18-72hrs.

OBSERVATION: No white color colonies were observed on the plate.

RESULT:

This test indicates the absence of staphylococcus aureus in the given water sample.



BIOCHEMICAL TESTS: Several biochemical tests are performed to confirm the presence of Staphylococcus aureus and are as given as follows:-

1. Coagulase test.
2. Gelatin Hydrolysis.
3. DNase Test.
4. Baird Parker Agar Test.

COAGULASE TEST: It is a test used to differentiate staphylococcus aureus (positive) which produce an enzyme called coagulase from S. epidermis which do not produce coagulase. It is a type of enzyme like protein that causes plasma to clot by converting fibrinogen to fibrin.

PROCEDURE: Coagulse can be detected by two different methods and are as follows:

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SLIDE TEST: About 10 micro litres of deionised water or saline is added to a slide. Inoculate loop full of mixture from fresh culture and are added to the water to obtain a smooth milk-suspension. Add a drop of rabbit/human plasma on the slide and leave for NMT 10 seconds.

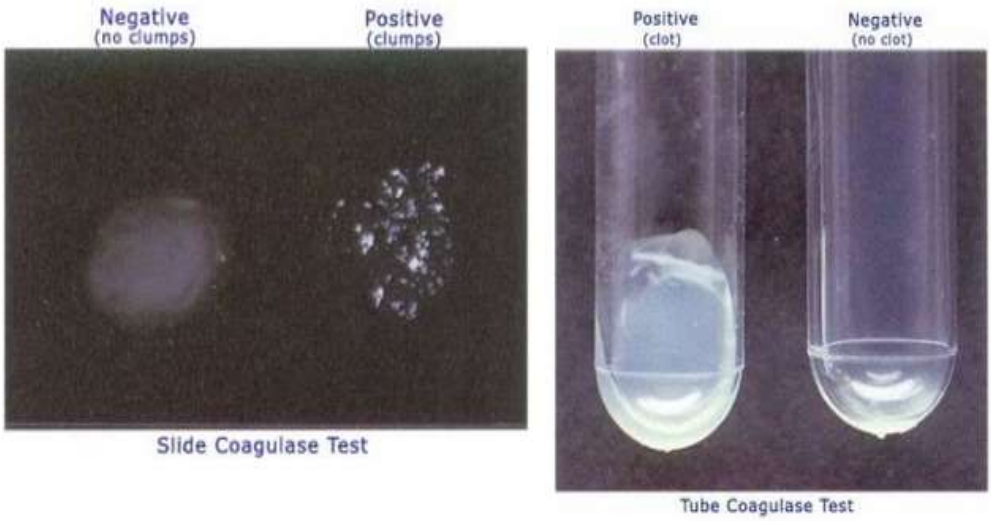
OBSERVATION: Clumping observes within 10 seconds after addition of plasma.

RESULT: The above test confirms the presence of coagulase positive organisms like Staphylococcus aureus.

TUBE TEST: The plasma is diluted with saline and this diluted sample is added to a test tube. Inoculate the loop full of culture to the test tube. It is completely mixed and incubated at 37C for an hour.

OBSERVATION: Clot formation occurs in the test tube before 24hrs.

RESULT: The above test confirms the presence of coagulase producing organism such as Staphylococcus aureus.



GELATIN HYDROLYSIS: It is a biochemical test performed as a presumptive test for the identification of gram positive microbes. This test detects the ability of bacteria to produce gelatinase. It aids in distinguishing between gelatinase positive and gelatinase negative organisms. Gelatin is a protein derived from the connective tissue of vertebrates. It is produced when collagen is boiled in water. Gelatinases are proteases secreted extracellularly by some bacteria and hydrolyze gelatin.The standard and most commonly employed method is Nutrient gelatin stab method which offers easy and accurate interpretation of results and eliminates the use of chemicals or reagents.

PROCEDURE: In this, add approximately 1 ml of water sample into the test tubes containing nutrient gelatin medium.These test tubes are now incubated at 25C for 48hrs. Gelatin normally liquefies at 28 C. To confirm that liquefaction is due to gelatinase activity, these tubes are immersed in ice water bath for 15-20 minutes. Then tubes are tilted to observe if gelatin is hydrolyzed or not.

OBSERVATION: Hydrolysis of gelatin by gelatinase is visualized in the liquid medium after exposure to the cold temperatures.

RESULT: The above test confirms the presence of gelatin producing organisms such as staphylococcus aureus.

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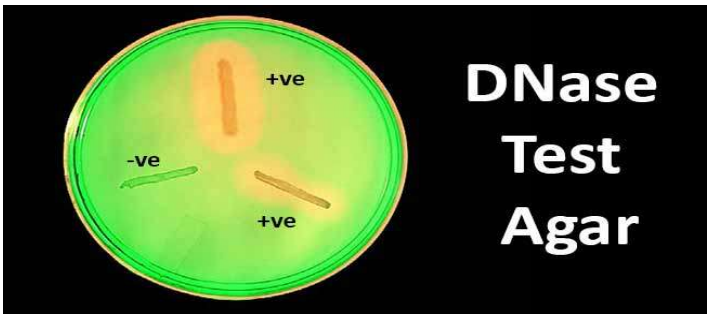
DNase TEST: It is the test used to determine the ability of an organism to hydrolyze DNA. DNase agar is a differential medium that tests whether the organism produce an exonuclease enzyme called deoxyribonuclease. DNase agar contains nutrients for the bacteria, DNA and methyl green as an indicator. Methyl green is a cation which binds to the negatively charged DNA. When the DNA is broken down, it no longer binds to the methyl green, and green colour fades away and the colony is surrounded by a colourless zone.

PROCEDURE: Dry the surface of agar plates before use. Each plate may be divided into sections by drawing lines over the bottom of the plate. Add loop full of the bacterial inoculums into the agar plates along with the DNase agar medium. Incubate the plates at 37 C for 18-24 hrs. After incubation, the plate is flooded with Hydrochloric acid which precipitates the polymerized DNA, making the medium opaque.

OBSERVATION: A clear zone is observed around the microbe.

RESULT: The above test confirms and it is identified as Staphylococcus aureus.



BAIRD PARKER AGAR TEST: It is used for the identification of coagulase positive staphylococci in food and pharmaceutical products. This test was developed by Baird Parker in 1962 and hence the name. Sodium pyruvate is added as a selective growth stimulant.

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PROCEDURE: Dry the surface of the agar plates for a minimal period of time prior to use. With a glass spatula, spread loop full of bacterial inoculum mixed with buffered peptone water and added on agar surface until dry. Incubate the inverted dishes at 35C. Examine after 24hrs for any colony observation on the plate.

OBSERVATION: A black, convex, shiny colonies of 1-1.5 mm in diameter were observed on the plates.

RESULT: The above test confirms and it is identified as Staphylococcus aures.

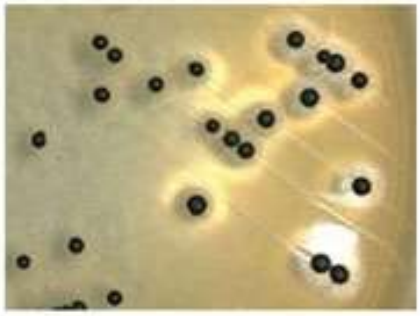

FIGURE 2: Formation of black colonies surrounded with clear zones indicating *Staphylococcus aureus* on the Baird-Parker agar.

SALMONELLA: It is a type of gram negative, non-spore forming, rod shaped, chemotrophic bacteria mostly cause digestive problems like diarrhea in humans .They invade blood stream and secrete endotoxins which cause septic shock and requires intensive care of the patients.

IDENTIFICATION OF SALMONELLA: Add 0.1ml of enrichment broth such as SCDB into 10ml of Rappaport Vassiliadis Salmonella Enrichment Broth (RVSEB) tubes. Incubate the tubes at 30-35C for 18-24 hrs. Subculture the loop full of suspension from RVSEB and streak (quadrant streak) on Xylose Lysine Deoxycholate Agar (XLDA) plate. Incubate the agar plates at 30-35C for 18-48hrs.

OBSERVATION: No red color colonies with black centre were observed on the plate.

RESULT: The above test indicates the absence of salmonella in the given water sample.

BIOCHEMICAL TESTS: Several biochemical tests were performed for identification of salmonella in the given sample and some of them are as follows:

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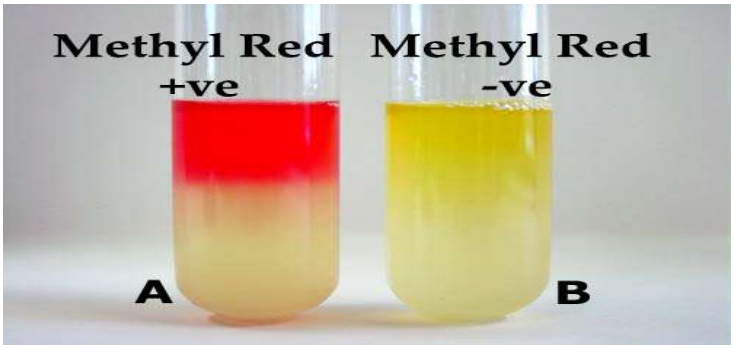
- Triple Sugar Iron Agar Test.
- H2S test.
- Methyl Red test.
- Urease test.

TRIPLE SUGAR IRON AGAR TEST (TSIA): It is a test designed to differentiate among the different groups or genera of gram negative bacilli that are capable of fermenting glucose by acid production.

H_2S TEST: It is a test used for the detection of hydrogen sulfide gas produced by an organism. This test is mostly used to assist enterobacteriaceae family. It is produced by certain bacteria through reduction of sulfur-containing amino acids such as cysteine, methionine etc.

METHYL RED TEST (MR): Organisms have an ability to produce a stable acid after glucose fermentation.Some bacteria produce large amounts of acids from glucose fermentation so that they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4.



UREASE TEST: It is a test used to identify those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is used to distinguish between urease positive and urease negative.

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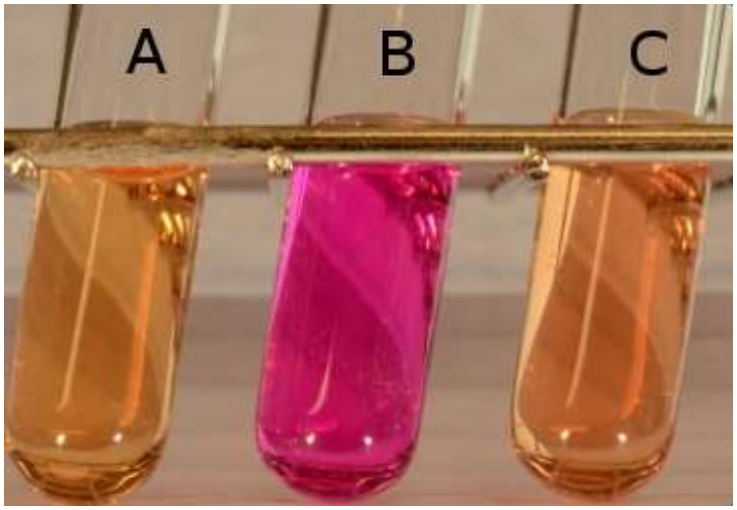
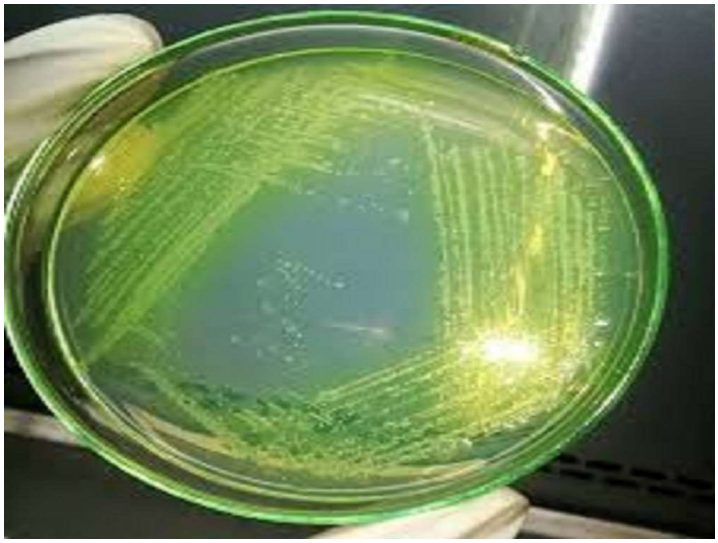


PSEUDOMONAS AERUGINOSA: It is encapsulated, gram-negative, facultative aerobe, rod-shaped bacteria. It is opportunistic pathogen causing affecting immune compromised patients. It is known as leading cause of morbidity in Cystic fibrosis patients and cause nosocomial infections. Due to survival and intrinsic resistance to multiple classes of antibiotics, infections of P. aeruginosa strains can be life threatening.

Their metabolic capacity is extensive as exemplified by their ability to produce secondary metabolites and polymers and also uses various carbon sources and electron acceptors. **IDENTIFICATION OF PSEUDOMONAS:** Add loop full of pre-enrichment broth on to the Cetrimide agar plates and are allowed to quadrant streaking. Incubate the plates by inverting and stored at 30-35C for 18-72hrs.

OBSERVATION: No formation of blue-green color colonies with metallic sheen on the agar plates.

RESULT: It indicates the absence of Pseudomonas aeruginosa in the given water sample.



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BIOCHEMICAL TESTS: These are the confirmatory tests used to identify the presence of Pseudomonas aeruginosa in the given sample.

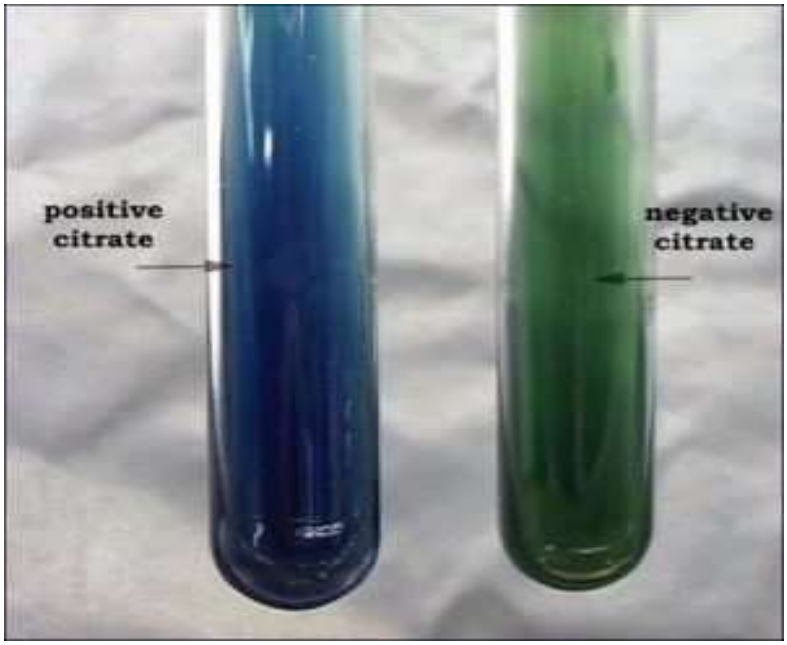
- Citrate Test.
- Beta Hemolysis Test

CITRATE TEST: It is a test used to determine the ability of bacteria to use sodium citrate as only carbon source and inorganic ammonium hydrogen phosphate as nitrogen source.

PROCEDURE: Consider a slant, inject Simmon's Citrate agar gently on the slant by slightly touching the tip of needle to the colony In citrate medium, organisms require more time to grow and are incubated at 35-37 C for 18-24hrs.

OBSERVATION: Change in color from green to blue indicates alkylation process.

RESULT: The above test will confirm the presence of Pseudomonas aeruginosa in the given sample.



BLOOD AGAR PLATE TEST (BAP): It is associated with complete lysis of red blood cells surrounding the colony. It is caused by two hemolysins, O and S, the former is inactive in presence of oxygen thus stabbing the plate increases the intensity of hemolysis reaction. S is an oxygen-stable cytotoxin.

PROCEDURE: Using a sterile inoculating loop, a loop full of inoculums is transferred on to the blood agar tube.Place the inoculated tube into the incubator and maintained at a temperature of 35-37 C.Incubate this test only for 24 hrs.

OBSERVATION: It is characterized by a clear (transparent) zone surrounding the colonies.

RESULT: The above test confirms the presence of Pseudomonas aeruginosa in the given sample.

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S.NO	TESTS	Escherichia coli	Salmonella	Pseudomonas aeruginosa	Staphylococcus aureus
1.	Catalase	+	+	+	+
2.	Indole	+	-	-	-
3.	Methyl red	+	+	-	+
4.	Voges-Proskaeur	-	-	-	+
5.	Citrate	-	-	+	+
6.	Coagulase	-	-	-	+
7.	Gelatin hydrolysis	-	-	+	+
8.	Urease	-	-	-	+
9.	Oxidase	-	-	+	-
10.	Hemolysis	+	-	-	+

TEST – II: BACTERIAL ENDOTOXIN TEST: Bacterial Endotoxin Test is an invitro assay where one is very keen to observe whether the given sample contain endotoxins or not. Endo means inside, toxin means harmful. As name itself indicates Endotoxin is a lipo polysaccharide which is present inside the cell wall of gram negative bacteria and releases out when the bacteria undergoes death or decline state. Endotoxins are also called as pyrogens that are fever causing agents in humans.

Gram-negative bacteria express various amphiphiles at their surface among which the lipopolysaccharides (endotoxins, O-antigens) have been studied most intensively. Lipopolysaccharides consist of a heteropolysaccharide portion (O-specific chain and core) which is responsible for the O- (and R-) antigenic properties and a covalently bound lipid component, termed lipid A, which contains the endotoxic principle of lipopolysaccharides.

LPS interacts with multiple components of blood, including various cellular elements, lipoproteins, plasma proteins, and vascular endothelial cells. Interactions of very low concentrations of LPS with elements of blood can have profound

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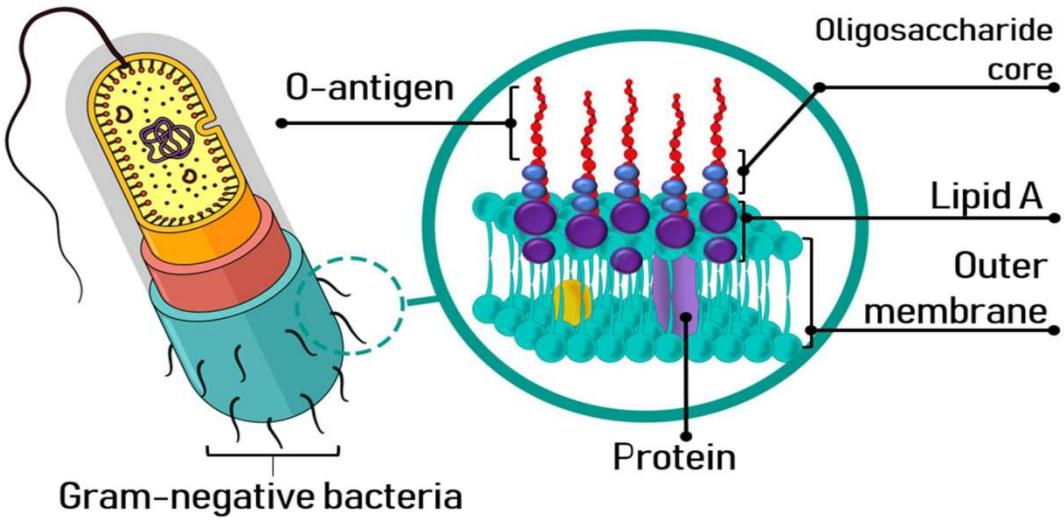
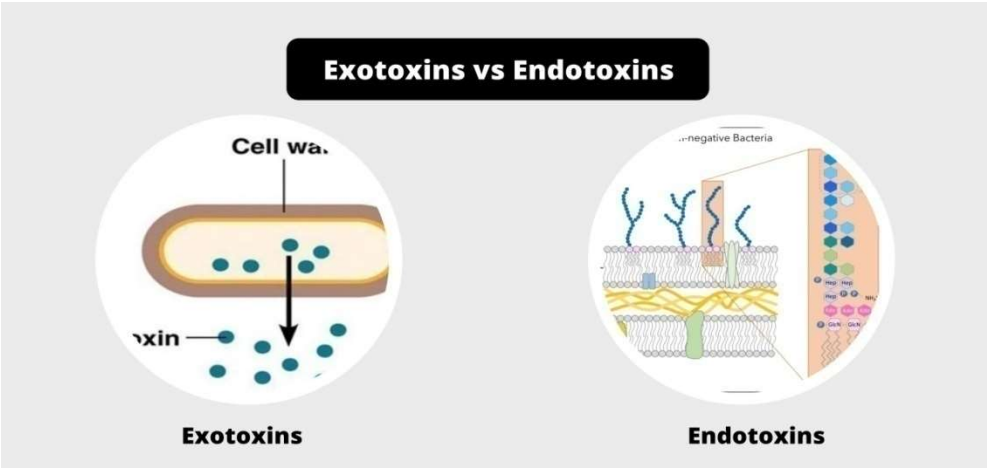
physiologic consequences, suggesting that these effects are likely to be receptor mediated. Recently, a growing number of mammalian endotoxin-binding proteins have been identified.



The Limulus assay can easily detect as little as 1 mg of bacterial endotoxin per ml of fluid after an assay period of less than 2 h. This assay is now recognized as the most sensitive test available for the detection of bacterial endotoxin. The test is simple, specific, rapid, and inexpensive compared to the USP rabbit pyrogenicity test. Since its introduction, the Limulus assay has been applied to the detection of endotoxin in a variety of fluids, including blood from patients suspected of gram-negative sepsis for the study of experimental endotoxemia and shock in laboratory animals for rapid detection of gram-negative bacterial meningitis for screening of urine for significant bacteriuria and as a method for detection of pyrogenic parenteral fluids.



In Pharmaceutical industry water is widely used as a main ingredient, raw material and solvent processing and in manufacture of pharmaceutical products. There are different grades of water like purified water which is used as an

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excipient in production of non–parenteral preparations and in other applications such as cleaning of equipments .These purified water must meet requirements of ionic and organic chemical purity and must be free of microbial contamination.

Characteristics of Bacterial Endotoxin:

"Microbial pyrogen" as opposed to "gram negative bacterial endotoxin" has become a general descriptive term for many different substances. However, pyrogenic substances can be produced by some gram positive bacteria, mycobacteria, fungi and also viruses, but the pyrogens produced by gram negative bacteria, i.e., the endotoxins, are of significance to the pharmaceutical industry.

Bacterial endotoxins, found in the outer membrane of gram-negative bacteria are members of a class of phospholipids called lipopolysaccharides (LPS). LPS are not exogenous products of gram negative bacteria. The release of LPS from bacteria takes place after death and lysis of the cell. Good examples of pyrogen producing gram- negative bacteria are Escherichia coli, Proteus, Pseudomonas, Enterobacter and Klebsiella.

GEL-CLOT METHOD: The Gel-Clot method is based on the presence or absence of a gel clot in your sample tube. The gelation occurs when proteins are coagulated due to the presence of endotoxins. Using the Gel-Clot method, the detection limit is normally between 0.01 and 0.03 endotoxin units per one milli litre of the solution used in the test. This means that a solid gel does not come to be formed below this concentration of endotoxins when moving the test tube. A criterion used in the method of gelation is to turn the test tube 180º and ascertain that the gel remains intact.

CALCULATION OF MAXIMUM VALID DILUTION (MVD): Maximum Valid Dilution is the maximum allowable dilution of a sample at which the endotoxin concentration is determined

$$\text{MVD} = \frac{\text{EL X POTENCY OF SAMPLE}}{\Lambda}$$

Where, EU = endotoxin limits/ml

Potency of sample=Conc. of product in mg/ml

Λ= Lysate sensitivity.

$$= \frac{1 \text{ X } 0.25}{0.03125}$$

$$= 8$$

ESTABLISHMENT OF ENDOTOXIN LIMITS: The effects of endotoxin are related to the amount of endotoxin in the product dose administered to a patient. Because the dose varies from product to product, the endotoxin limit is expressed as K/M. K is 5.0 EU/kilogram (kg), which represents the approximate threshold pyrogen dose for humans and rabbits. That is the level at which a product is adjudged pyrogenic or non-pyrogenic. M represents the rabbit pyrogen test dose or the maximum human dose per kilogram that would be administered in a single one hour period, whichever is larger.

RECONSTITUTION OF LYSATE REAGENT: Store and reconstitute the lyophilised reagent with LRW water which is frozen and thawed only once as per the manufacturer. Reconstitute lyophilized lysate by adding 5.2 ml LAL Reagent Water to the 50ml of test vial. Swirl gently but thoroughly for at least 30 seconds. Do not shake as contents will foam.

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RECONSTITUTION OF CONTROL STANDARD ENDOTOXIN REAGENT: Reconstitute the CSE with known amount of LRW solution and stored as per manufacture. Vortex atleast for 5 minutes as before using atleast one minute before.

CONFIRMATION OF LABELLED LYSATE SENSITIVITY: Confirmation tests must be carried out when a new batch of lysate is used when there is any change in experimental conditions which may affect the outcome of the results. Prepare CSE dilutions of 4Λ of atleast 4 concentrations equivalent to2Λ, Λ, 0.5Λ, 0.25 Λ by diluting a series of CSE solutions with LRW water.

CSE DILUTIONS:

TUBE NUMBER	VOLUME OF LRW (ml)	VOLUME OF CSE(ml)	TOTAL VOLUME(ml)	CSE FINAL CONCENTRATION
01	1.8	0.2	2.0	100
02	1.8	0.2	2.0	10
03	3.6	0.4	4.0	0.1
04	2.0	2.0	4.0	0.5
05	2.0	2.0	4.0	0.25
06	2.0	2.0	4.0	0.125

Incubate the reaction mixtures by transferring the test tubes into heating block and maintain temperature at 37±1 for 60±2 minutes avoiding vibration. After incubation take each test tube and directly invert it through approximately 180degrees in one smooth motion. If a firm gel has formed upon inversion record the result as positive and if no intact gel is formed it is recorded as negative. The test is not valid unless the lowest concentration of the standard solutions shows a negative result in all replicate tests. The endpoint is the last positive test in the series of decreasing concentrations of endotoxin.

Calculate the mean value of the logarithms of the endpoint concentration and then the antilogarithm of the mean value using the following equation:

Geometric Mean Endpoint Concentration = antilog (Σe / f),

Where Σe= the sum of the log endpoint concentrations of the dilution series used .

f = number of replicate test tubes.

The geometric mean endpoint concentration is the measured sensitivity of the LAL Reagent (in EU/mL). If this is not less than 0.5Λ and not more than 2Λ, the labeled sensitivity is confirmed and is used in tests performed with this lysate.

ASSAY SCHEME:

TUBE NO	TEST	VOLUME OF LRW	VOLUME OF CSE	LAL
1,2	NWC	100	NA	100
3,4,5,6	2Λ	NA	100	100
7,8,9,10	Λ	NA	100	100
11,12,13,14	Λ/2	NA	100	100
15,16,17,18	Λ/4	NA	100	100

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OBSERVATION AND CALCULATION:

REPLICATIVE NO	NWC	2Λ	Λ	Λ/2	Λ/4	END POINT (A)	LOG_{10} END POINT (B)	MEAN OF LOG_{10} END POINTS	GEOMETRIC MEAN END POINT
1	-	+	+	-	-	0.03	-1.522		
2	-	+	+	-	-	0.03	-1.522	-1.522	0.03
3	NA	+	+	-	-	0.03	-1.522		
4	NA	+	+	-	-	0.03	-1.522		

- \- = No gel formation.
- \+ = Gel Formation.
- NWC = Negative Water Control.

ACCEPTANCE CRITERIA:

"-''TWO FOLD VALUE= (Λ/2)	"+" TWO FOLD VALUE = (2Λ)
0.015	0.06

REMARKS: The observed geometric mean end point of CSE dilution series is within the two fold of labelled LAL sensitivity of lysate.

TEST PROCEDURE: Prepare the solutions as given in the following format:

SOLUTION	CONTROLS	LRW (µl)	CSE (µl)	WATER SAMPLE	LYSATE (µl)	No. of Replicates.
A	NPC	50	-	50	100	2
B	PPC	-	50	50	100	2
C	PWC	50	50	-	100	2
D	NWC	100	-	-	100	2

Where,

NPC = Negative Product Control.

PPC = Positive Product Control.

PWC = Positive Water Control.

NWC = Negative Water Control.

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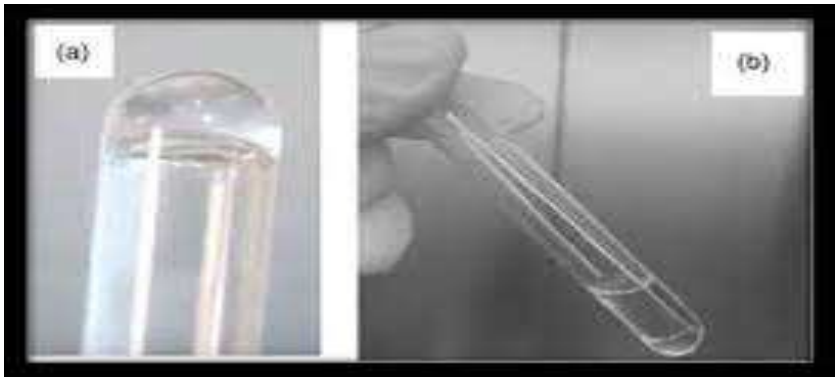
- Mix the assay tubes by vortexing and incubate in a heating block where the temperature is maintained at 37± 1C for 60 ± 2minutes.

ASSAY AND OBSERVATION:

TUBE NO	TEST CONTROLS	LRW	CSE	LAL	OBSERVATION I	OBSERVATION II
1,2	NWC	100	NA	100	-	-
3,4	PWC	50	50	100	+	+

DISCUSSION: The formation of firm gel capable of maintaining its integrity when the test tube is inverted to 180 is indicated as positive test. A negative test is characterized by the absence of gel or formation of viscous mass when inverted the tube at 180. The test is not valid unless both replicates of two solutions B and C are positive and solution D are negative. Repeat the test when if a positive result is found for one replicate and negative is found for other replicate.



ACCEPTANCE CRITERIA:

- ❖ NWC= BOTH REPLICATES MUST BE NEGATIVE.
- ❖ PWC = BOTH REPLICATES MUST BE POSITIVE.

It indicates that the endotoxins are not present in the given water sample.

CONCLUSIONS: The Harmonized Microbial Limits test is the globally standardized method for determining the bioburden of a given raw material or final product formulation of oral solid non-sterile product. It will continue to be used in industry as defined by compendia to release product for human use. Microbial limit test has to be performed with extreme vigilance as minute of the sample contamination affects the quality of the sample and the conclusion of the test These applications require pharmaceutical grade water to be used, which is water that has been through a chemical purification step. Purification is undertaken so that the water is free of substances that might cause interaction with drug substances, as well as to obtain water of an appropriate microbiological standard .Microbiological risks are ever present with water systems. Risks can arise through poorly maintained water generation systems, through badly designed distribution networks (such as the presence of dead legs), or at user outlets (where ineffective tubing management can lead to contamination). Weaknesses in water systems are exacerbated by microorganisms being ubiquitous and varied in their ability to survive. Therefore, monitoring pharmaceutical grade water system for bioburden is important. In this study, we have shown that it is possible to analyse the concentration level of bacterial endotoxin in purified water using

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the gel-clot method and no gelation was observed in the negative product control. . At the same time, this study can be regarded as a supplement analysis method for WFI as per United States Pharmacopiea 85 (USP).

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