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Microbial analysis of water system for veterinary vaccine manufacturing facility

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Abstract
Water is very widely used in the pharmaceutical, biopharmaceutical and biological industry for various purpose like as raw material, ingredient as a solvent in processing, formulation and manufacturing of the wide range of the finish products, as API and intermediates and analytical reagents. That's why regulatory requirements over control of the quality of various types of the waters throughout the production, storage and distribution processes, including microbiological and chemical quality, are very stringent. Lack of monitoring over any of the parameters for the Potable, Purified or Water for Injection can leads to the loss of the costly drug product or drug substance.
Veterinary vaccines are considered as the sterile parenteral dosage form and water used for the various purposes in the veterinary vaccine manufacturing facility must have to comply with the regulatory requirements. So the aim of the present work is to bioburden testing and check the suitability of the different types of the water used for the various applications in vaccine manufacturing facility using the various techniques like MPN method, Plate count method, Filtration method and based on the results of the bioburden testing and identification of pathogens and conclusion to be done for the compliance and suitability of the water used for the manufacturing of the vaccines as sterile parenteral dosage form to have the claimed safety, integrity, strength, purity and quality.

Keywords: Water for injection, purified water, potable water, MPN, plate count, filtration method

1. Introduction
Water must follow quality needs as stated in principles given by the United States Pharmacopeia (USP), European Pharmacopoeia (EP) before its usage in pharmaceutical manufacturing (Kolpin *et al*., 2002) [1].
Due to its criticality in pharmaceutical production, microbiological control of water is very important. Since water is ever present, each grade of water is a potential wellspring of microbiological contamination, particularly when not appropriately controlled (Sandle *et al*., 2004) [2]. The nature of protected water sources can be disintegrated because of poor site choice, lacking assurance and unhygienic management of facilities. Water may become contaminated at any point in between its collection and use.
Despite the fact that most pharmaceutical water frameworks are controlled, microorganisms will once in a while be available, although in low numbers (Jiminez *et al*., 2004) [3]. A few of the microorganisms noted will be found in pharmaceutical water generation plants, starting from the supplied potable water. It is evaluated that there are 70 distinct kinds of bacteria in wastewater. A few unique kinds of microbes cross water-treatment barriers and are found in pharmaceutical waters. Most microbial contaminants are Gram-negative bacteria that represent the extra risk of Endotoxin contamination of waters utilized for parenteral production (Traeger *et al*., 2003) [4].
To check whether the water is contaminated with the microorganisms or not different types of microbiological test are performed to check the purity of water such as membrane filtration method, plate count method, most probable number etc. Microbial conditions are usually checked by experimental method that need as much as 48 to 72 hours for giving results. (Twinbrook 2012) [5].
Among the microorganisms generally found in water, these organisms are Mainly Gram-negative bacteria. Common species include: *Pseudomonas aeruginosa, Salmonella* spp,

Staphylococcus aureus and *Escherichia coli*. Very less gram-positive bacteria are isolated those that are identified include *Bacillus* spp. Validation and qualification of water purification, storage and distribution system are a fundamental part of GMP and form an integral part of the GMP inspection.

Water in pharmaceutical industry is purified first by various processes before using it for any operation. The treatments for purification of water include using of softeners, ultrafiltration, reverse osmosis, distillation etc. Water which is obtained after passing through all these treatments is completely pure and sterile which does not contain any kind of microbial contamination (Murthy *et al*., 1984) [6].

If appropriate steps are not taken to lessen their numbers or eliminate them, these microorganisms may compromise subsequent water purification steps. Endogenous sources of microbial contamination can arise from unit operations if in a water purification system is not properly maintained and operated.

2. Materials and Methods

Sample collection: While collecting bulk water at the points of use first allow a forceful flow of water for about 1-3 min before sampling. Approx. 100 ml of sample is collected in the bottles form the quality control lab and they are covered by aluminium foil immediately to prevent any external contamination. Samples are processed for testing within one hour after collection to obtained most accurate result. Types of Water Samples to be tested are as below.

o	Non-potable Water
o	Potable water
o	Purified Water
o	Water for Injection

2.1 Membrane Filteration (MF) Technique

Filtration assembly having six filtration cup together is sterilised to avoid any unwanted contamination. Sterile filtration assembly is set up inside the laminar air flow. Using a sterile forceps, a sterile membrane filter with a rating of 0.45µm is placed under the filtration cup on the porous plate. The filter unit is connected to a suitable vacuum pump. The vacuum motor pump is switched on to draw the samples through the filter. A fixed volume of water is then added into the filtration cup. Upon completion of filtration microorganisms are retained on the surface of the membrane. After the filtration, the funnel is unlocked and removed. The membrane filter is removed with sterile forceps and placed on the SCDA plate in the centre carefully to avoid the entrapment of air. The SCDA plates are incubated at 37°C for 24 hours. On the next day observe the plates and count the colonies.

2.2 Most Probable Number (MPN) Method

2.2.1 Preparations for presumptive test

Single strength broth and double strength broth are prepared. Double strength broth contains double the concentration of ingredients except water. Prepare five tubes of MLBB having 10 ml (2x) medium with 10 ml of sample in each. Another set of five tubes of MLBB having 5 ml (x) medium with 1 ml of sample in each. Prepare third set of five tubes of MLBB having 5 ml (x) medium with 0.1 ml of sample in each.

2.2.2 Presumptive test

First of all shake the water sample to ensure uniform distribution of microorganisms. Prepare MacConkey media of single and double strength in sterile test tubes with Durham's tube and autoclave it. Prepare three sets of test tubes having five tubes in each set, one set with 10 ml of double strength and other two having five ml of single strength. One tube having five ml of medium is left uninoculated, and marked it as control tube. Using sterile pipettes and transfer 10 ml of water to each tubes of double strength broth. Transfer one ml of water sample to each of the five tubes of one set of single strength broth and transfer 0.1 ml water to five tubes of remaining last set of single strength broth tubes. Incubate the tubes at 37 °C for 24 hours. After incubation, observe the tubes for gas production which is collected in Durham's tube and acid production is indicated by colour change of media from pink to yellow. If none of the tube show acid and gas production then reincubate all tubes for additional 24 hours. On the next day, observe and record the number of positive tubes in each set and compare with standard MPN index to give presumptive coliform count per 100 ml water sample.

2.2.3 Confirmed test

From positive presumptive tubes spread 0.1ml of sample on EMB agar plate. Incubate one plate at 37 °C for 24 hours and another at 44.5 °C for 24 hours.

2.2.4 Completed test

Select and mark a well isolated typical or atypical colony from the EMB agar plate. With the help of sterile nicrome wire loop, pick half of the previously marked typical/atypical colony and transfer it to BGLB broth tube. From the remaining half of the same colony streak on the surface of a nutrient agar slant. Incubate slant and broth at 37 °C for 24 hours. Check lactose broth for presence of acid and gas. Prepare Gram's stain of the growth from the surface of agar slant and observe the slide. Look for the presence of gram-negative non-spore forming short rods. Record the result and interpreted.

2.3 Pathogen Identification Testing

2.3.1 Test for identification of *Escherichia coli*

Take a loopful of sample from each positive SCDA broth and spread it on a EMB agar plate. Incubate the plate at 37° C for 24-48 hours. If the colonies exhibit greenish metallic sheen under reflected light and blue black appearance, then it confirms the presence of *E. coli*. Upon examination, if none of the colonies exhibits both a characteristic metallic sheen under reflected light, the sample meets the requirements of the test for the absence of *E. coli.*

2.3.2 Test for identification of *Salmonella* spp.

Take a loopful of sample from positive SCDA broth and spread it on a xylose-lysine-deoxy cholate agar (XLDA) plate and brilliant green agar (BGA) plate. Simultaneously carry out the positive control by streaking a loop full growth of *salmonella* on a surface of one of the above media, which is used for testing. For negative control incubate the agar plate without streaking or inoculation. Invert and incubate all the plates at 37° C for 18-24 hours. If the colonies appear small, transparent, colourless or pink colonies with pink or red zone on BGA plate, then it confirms the presence of *Salmonella.* On XLDA plate, colonies appear Red with or without black centers.

2.3.2.1 Secondary Test

Subculture any colonies showing the above characteristics in triple sugar- iron agar by first inoculating the surface of the slope and then making a stab culture with the same inoculating needle. At the same time inoculate a tube of urea broth. Incubate at 36 ºC to 38 ºC for 18-24 hours. Upon examination, no evidence of tubes having alkaline (red) slant and acid (yellow) butt (with or without concomitant blackening of the butt from hydrogen sulphide production), the sample meets the requirements of the test for absence of genus salmonella.

2.3.3 Test for identification of *Staphylococcus aureus*

Streak a portion from Soyabean casein digest medium on the surface of mannitol salt agar medium. Simultaneously carry out the positive control by streaking a loop full growth of *Staphylococcus aureus* on the surface of agar medium. For negative control incubate the agar plate without inoculation. Invert and incubate all the plates at 37 ºC for 18 to 24 hours. If upon examination, if the plate contains colonies having yellow colonies with yellow zones, then it indicate the presence of *Staphylococcus aureus.*

2.3.4 Test for identification of *Pseudomonas* spp.

Streak a portion from Soyabean casein digest medium on the surface of Citramide agar medium; simultaneously carry out the positive control by streaking a loop full growth of *P.aeruginosa* on the surface of Citramide agar. For negative control incubate the Citramide agar plate without inoculation. Invert and incubate all the plates at 35 ºC to 37 ºC for 18 to 24 hours. If upon examination, if the plate contains colonies having greenish yellow growth, the sample indicates the presence of *Pseudomonas aeruginosa*.

2.3.4.1 Confirmative Test

The suspected colonies from Citramide agar plate are smeared on the Oxidase disc. The test is positive, if purple colour is produced within 5-10 seconds.

2.4 Plate Count Method

Prepare a series of at least 3 test tubes containing 9 ml of sterile distilled water. Using a sterile pipette, add 1 ml of sample in the first tube of the set and label it as $10^{-1.}$ Mix the contents well by swirling the tube upside down few times. From the first tube, take 1 ml of the sample and transfer to second tube. Label it as $10^{-2.}$ Repeat the procedure with all the remaining tubes labelling them until $10^{-3.}$ Pipette out 0.1 ml from the appropriate desired dilution series onto the center of the surface of an agar plate. Spread the sample evenly over the surface of agar using the sterile glass spreader. Incubate the plate at 37 ºC for 24 hours. Calculate the colony forming unit (CFU) value of the sample.

Calculation of result:

$$\text{CFU/ml} = \frac{\text{(No. of colonies) X (Dilution factor)}}{\text{Volume of culture plated}}$$

3. Results

Table 1: MPN method

Water samples	Combination of positive tubes	MPN Index per 100 ml
Non Potable Water	5 3 3	180
Potable Water (Generation)	4 4 3	54
Potable Water (User Point)	4 2 3	38
Purified Water (Generation)	0 0 0	0
Purified Water (User Point)	0 0 0	0
Water for Injection (Generation)	0 0 0	0
Water for Injection (Use Point)	0 0 0	0

Table 2: Plate count method

Dilution	No of Colony			CFU/ml		
	10^1	10^2	10^3	10^1	10^2	10^3
Non Potable Water	298	270	189	2.98×10^4	2.70×10^5	1.89×10^6
Potable Water (Generation)	127	87	62	1.27×10^4	8.7×10^4	6.2×10^5
Potable Water (User Point)	102	85	43	1.02×10^4	8.5×10^4	4.3×10^5
Purified Water (Generation)	78	58	33	7.8×10^3	5.8×10^4	3.3×10^5
Purified Water (User Point)	53	42	31	5.3×10^3	4.2×10^4	3.1×10^3

3.1 Membrane Filtration Method

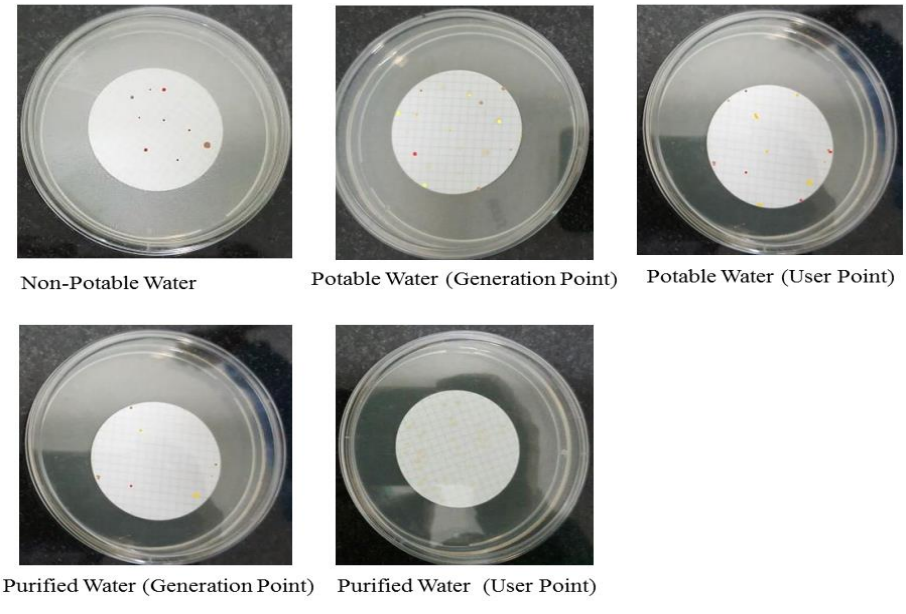
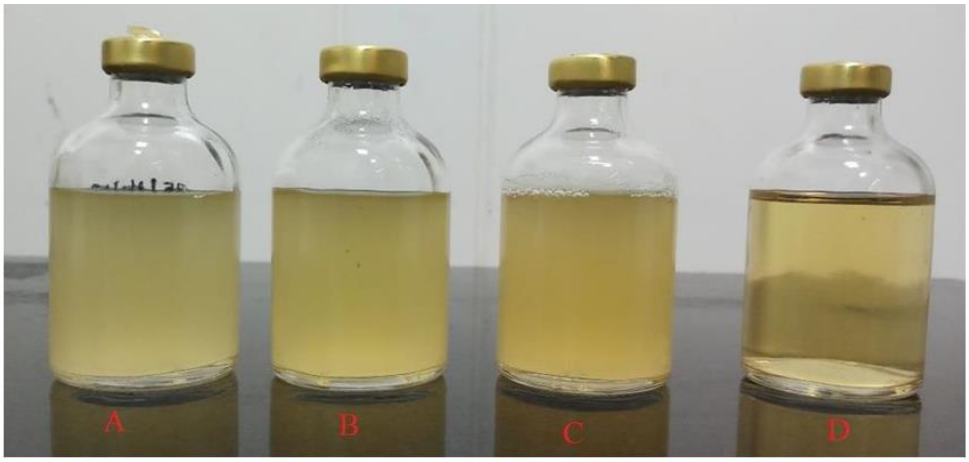


Fig 1: Observation of plates in the Membrane filtration method

3.2 Pathogen Identification Test



(A)Non-Potable Water, (B) Potable Water (Generation point), (C) Potable Water (User point)
(D)Purified Water (Generation point)

Fig 2: Enrichment of pathogens in SCDM broth

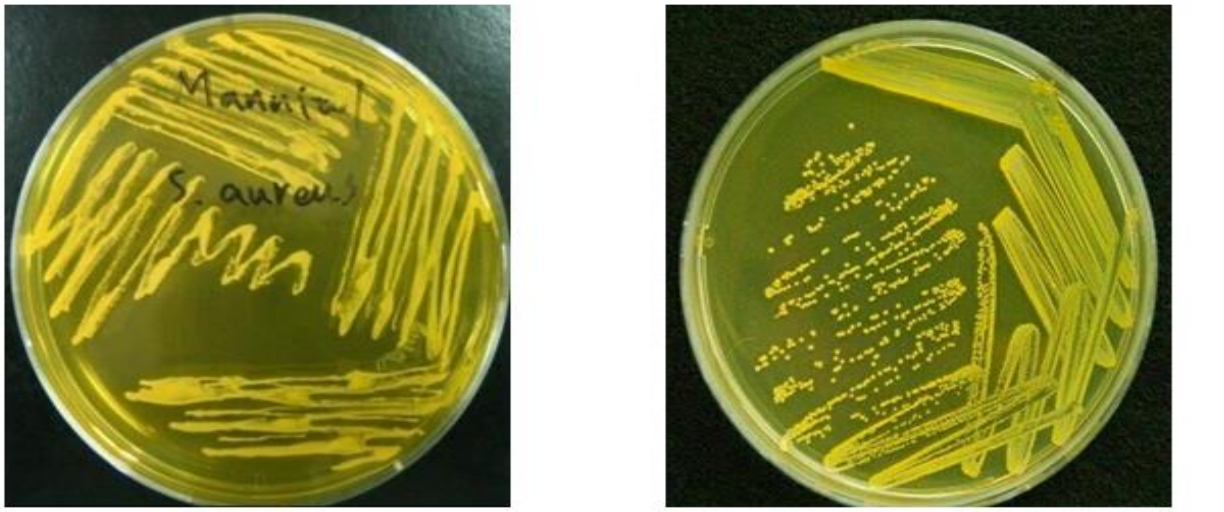


Fig 3: Isolation of *Staphylococcus aureus* on Mannitol Salt Agar

Fig 4: Isolation of *Salmonella abony* on XLDA Plate

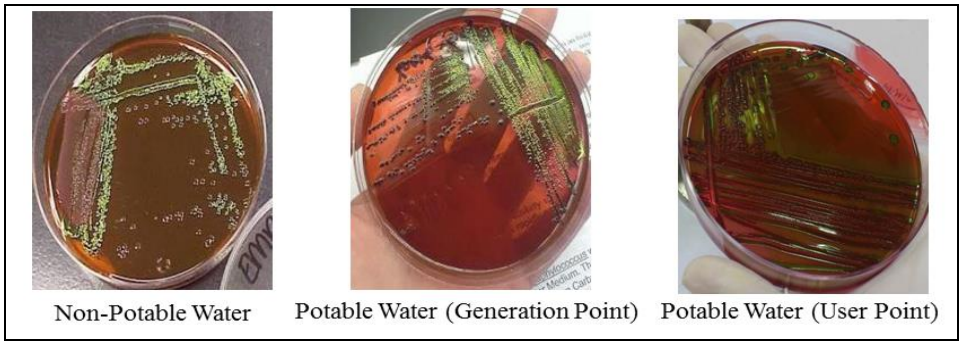


Fig 5: Isolation of *E. coli* on EMB agar

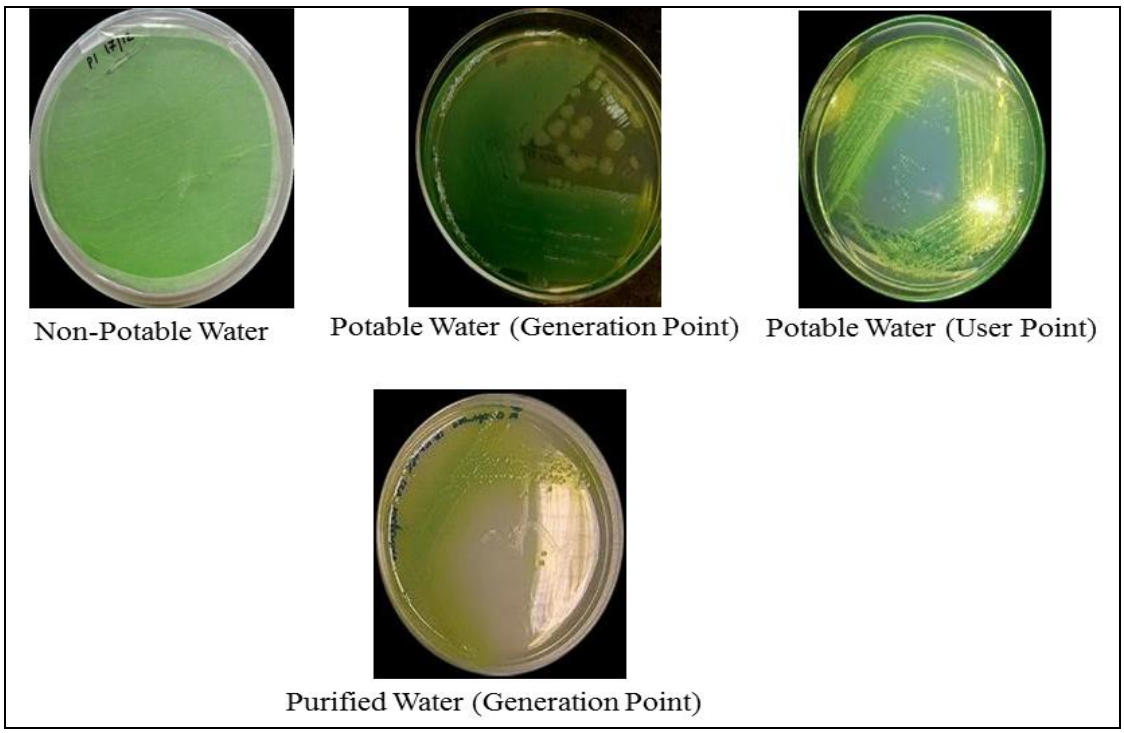


Fig 6: Isolation of *Pseudomonas aeruginosa* on Citramide agar

4. Discussion

The study result suggests that the common pathogenic microorganisms which get identified are commonly *E. coli*, *Salmonella abony*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

On EMB agar plate *E.coli* produce Greenish metallic sheen under reflected light while on Mannitol salt agar plate *S. aureus* produce yellowish colonies. *Ps. aeruginosa* produce greenish yellow pigment on citramide agar plate.

When a well isolated colony from citramide agar plate is transferred to oxidase disc it gives purple colour within five seconds. This confirms the presence of *pseudomonas.*

There is an absence of any pathogenic organisms in WFI and purified water in both the samples from generation and user point. On performing Membrane filtration method, each bacterium gets trapped on the filter which is then grows into a separate colony.

5. Conclusion

Bioburden testing of the Potable water, Purified water and Water for injection used for the manufacturing of the veterinary vaccines at the different stage of the process done for the generation points as well as the user points to be done using the MPN method, Plate count method and Filtration method. Identification of the pathogens was also done for the Potable water, Purified water and Water for injection used for the manufacturing of the veterinary vaccines. Results of Bioburden using all the methods shows that the bioburden limit for purified and water for injection are well within acceptable regulatory limits. So as far as the microorganisms are concerned based on the testing and its respective results reveals that Potable water, Purified water and Water for injection used for the manufacturing of the veterinary vaccines comply with the acceptance limit of the microbiology and states the control for the microorganism in water system as suitable for the manufacturing of the vaccines as sterile parenteral dosage form to have the claimed safety, integrity, strength, purity and quality.

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