ORIGINAL ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

A Comparative Study of the Phenotypic and Molecular Techniques for Identification of Pathogenic Gram-Negative Bacteria in Pharmaceutical Water

Marwa M. Azab* †, Sarah Shabayek† Aya G. Akl, Ali A. Abdelrahman

Department of Microbiology and Immunology, Faculty of Pharmacy, Suez Canal University, Ismailia, 41522, Egypt.

Received on: 11-03-2023

Revised on: 27-06-2023

Accepted on: 04-07-2023

*Correspondence Author:

Phone: +201024299630

E-mail address:

Marwaazab2515@yahoo.com

† These authors have contributed equally to this work

Abstract

Rapid and accurate identification of pathogenic bacteria is a fundamental goal in microbiological monitoring of pharmaceutical water quality, but one that is very difficult for many slow-growing and fastidious microorganisms. Purified water is widely used in pharmaceutical manufacturing in washing of equipment, preparation of finished product, and it is also used for rinsing equipment or for the preparation of disinfectants and detergents. A total of 150 purified water samples were collected from all the plant's departments over a period of 3 months from December 2020 to February 2021. Each sample was filtered on 0.45µ membrane filter then cultured on R2A agar plates. Bacterial isolates from water samples were morphologically categorized based on shape, size, colour, and its characteristic growth on selective media and biochemical testing. Colonies with different colors and morphologies were subjected to amplification and sequencing of a 1000–1500 nt portion of the 16S rRNA gene. A total of 235 colonies were isolated. These were categorized into 29 isolates as colonies showing identical morphology were grouped together. 16S rRNA Sanger sequencing categorized these 29 isolates into 18 genera and 22 species. Sixty-six colonies (categorized into 7 isolates) were presumed to the genus *Pseudomonas* by conventional method. However, 16S rRNA Sanger sequencing indicated 21 out of these 66 colonies (categorized into 2 isolates) belonged to another genus. For the purified water system, *Pseudomonas* was the most common gram-negative genus isolated, followed by *Enterobacter* then *Klebsiella* and *Citrobacter.*

Keywords: Pharmaceutical water monitoring, Conventional methods, 16S rRNA gene, *Enterobacter*, *Pseudomonas, and Citrobacter.*

1. Introduction

Water quality for human consumption is regulated by the European pharmacopeia, USP and International (ICH or WHO) GMP issues, engineering guides, or other regulatory (FDA, EPA, or WHO) guidance for water. In both types of water, an absence of fecal bacterial indicators is an indicator of safety, and heterotrophic bacteria are enumerated to manage water quality **(Bartram *et al*., 2003 & Chowdhury, 2012).** Traditional methods are the reference for the control of microbiological quality of water as they are reliable, easy to use and allow microorganisms identification. However, these methods are time-consuming and labor intensive **(Nemati *et al*., 2016)**. For many slow

growing and fastidious organisms, traditional phenotypic identification is difficult and time-consuming **(Cloud JL, *et al.*, 2010)**. They depend on the ability of microorganisms to show visible colonies after an incubation period of typically 3 days that can go up to 14 days. **(United States Pharmacopeia).** The long time-to-result is a concern for industries as improvement in processes and products requires faster methods to control microbiological quality. Therefore, over the past 25 years, many technologies have been developed to reduce the time-to-result. The culture-dependent approaches have been successful at gathering bacterial presence data; however, they have been criticized for their inability to accurately characterize the microbial diversity in natural environments **(Amann *et al*., 1995).** Besides, when phenotypic methods are used to identify bacteria, interpretation of test results involves substantial subjective judgement. Genotypic identification is emerging as an alternative or complement to established phenotypic methods **(Cloud JL, *et al*., 2010)**. Typically, genotypic identification of bacteria involves the use of conserved sequences within phylogenetically informative genetic targets, such as the small subunit 16S rRNA gene (**Clarridge III, J. E. (2004**). Consequently, molecular surveys based on 16S rRNA template as the target molecule may be useful to identify active bacterial contaminants **(Felske *et al*., 2000; Morgan *et al.*, 2002).** The aim of the current study was to evaluate the efficiency of 16S rRNA Sanger sequencing in comparison to conventional culture and biochemical testing in the detection and identification of pharmaceutical water contaminants.

2. Materials and methods

2.1. Samples and sampling conditions

From December 2020 to February 2021, the water samples were collected aseptically in sterile bottles with cap. Each the bottle was labelled with full details and properly handled till delivered to Microbiology laboratory and processed within two hours after collection. One hundred and fifty water samples were taken from different points of use. The samples were analyzed by membrane filtration method using R2A Agar as nutritive media **(Reasoner *et al.*,1985),** and incubated at 37^{o}C for 24-48 hrs. Separate colonies were then submitted to identification with conventional subculturing culturing on selective and differential media combined with conventional biochemical testing. Further identification was done using 16S rRNA Sanger sequencing **(Revetta *et al.*, 2010).**

2.2. Identification of bacterial isolates

2.2.1. Conventional method

Separate colonies from R2A agar were subcultured on Cetrimide agar, Endo agar, MacConkey's agar and Eosin Methylene Blue (EMB) then incubated at 37^{o}C for 24-48 hrs. Plates were examined for colony morphology where identical colonies were examined with Gram staining and then identification of bacterial contaminants was performed according to standard microbiological techniques using selective and differential media in addition to a battery of biochemical analysis tests as described by **(Sandle, T., 2016**). The biochemical tests included amylase test, catalase test, citrate test, gelatin test, indole test (Kovacs' reagent**)**, nitrate reduction test, oxidase test, sugar fermentation test, urease test and methyl red test (Voges Proskauer test) **(Table 1).**

2.2.2. Molecular identification of bacterial isolates using 16S rRNA Sanger sequencing

2.2.2.1. DNA Extraction

Extraction of DNA from bacterial isolates was done as per the protocol described by **Atashpaz *et al. 2008*** Briefly, a single colony was inoculated in nutrient broth and was grown for 24 hr. at 37 °C. From the 5 ml of culture, the cells were harvested and 800 µL of lysing buffer (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 1% PVP, 20 mM Na2EDTA and 0.2% LiCl) was added to the sample and incubated at 65 °C for 30 min. The sample was then centrifuged at 10000 rpm for 5 min at 4 °C. Equal volume of chloroform – isoamyl alcohol (24:1 v/v) was added to the supernatant, mixed and centrifuged at 12000 rpm for 8 min at 4 °C. The DNA was extracted from the aqueous layer by adding ice cold (−20 °C) isopropanol. The dried DNA pellet was dissolved in 50 µL of 1X Tris EDTA buffer. The quality and intactness of the extracted DNA was checked by running on 1% agarose gel. The A260/A280 absorbance ratio was used to determine undesired contaminations.

2.2.2.2. PCR amplification and sequencing of 16S rRNA genes.

PCR amplification and sequencing of the extracted DNA samples was done by Yaazh Genomics, Tamil Nadu kit. Amplification was done using the 16S rRNA universal primers described by ***Lane 1991***: Forward primer 27 F AGAGTTTGATCMTGGCTCAG and Reverse primer 1492 R TACGGYTACCTTGTTACGACTT**.** The PCR reaction was performed using MJ Research Peltier Thermal Cycler with the following conditions: Initial denaturation was done at 94 °C for 2 min, followed by 35 amplification cycles at 94 °C for 45 s, annealing temperature of primers was 55 °C for 60 s, and extension at 72 °C for 60 s. Final extension was done at 72 °C for 10 min **(Chagnaud *et al.*, 2001**). The resulting PCR products were purified using Montage PCR Clean up kit (Millipore) then handled using ABI

Table 1: The Biochemical tests used in microbial analysis of pharmaceutical water

Biochemical testing	Reference	Observation	Type of bacteria
Kovacs' reagent: (para dimethyl amino bezaldehyde + isoamyl alcohol + sulphuric acid)	Macwilliams, m. P. (2012)	Appearance of pink colored ring	Presence of *E. coli*
Methyl red (Voges Proskauer test	McDevitt, s. (2009)	Appearance of pink colored ring in methyl red	Presence of *E coli* and *Citrobacter freundii*
Citrate utilization test: (simmon's citrate medium + bromo thymol indicator	MacWilliams, M. P. (2009).	Appearance of green colour or blue colour in the medium Green Negative. Blue- Positive	Absence or presence of *Citrobacter freundii.*
Urease test: (urease is digested by urease enzyme resulted in release of ammonia)	Brink, b. (2010).	Appearance of yellow colour shows negative. Appearance of pink colour positive	Presence of *Citrobacter freundii.* and *Klebsiella pneumoniae*
Oxidase reaction: (tetra methyl parapholene diamine dihydro chloride)	Shields, p., & cathcart, l. (2010).	Appearance of purple colour within 30 seconds.	Presence of bacteria contains cytochrome oxidase like *pseudomonas aeruginosa*
Triple sugar iron test (TSI) (glucose, lactose and sucrose 1:10:10).	Lehman, d. (2005).	Black colour change in the media.	Presence of hydrogen sulphide gas producing bacteria.

PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems) and submitted for Sanger sequencing.

3. Results and Discussion

Among 150 samples analyzed, 235 different colonies were obtained. All the colonies were streaked on selective and differential media **(Figure 1).** Bacterial colonies from water samples were morphologically categorized based on shape, size, color, and growth on selective and differential media. Identical colonies were grouped into isolates. The total number of isolates after classification was 29 different isolates. Isolates were examined by PCR using universal 16S rRNA primers. A clear band of 1500 bp in length was obtained for each isolate (**Figure 2**). The 16S rRNA sequence for each isolate compared with NCBI database (https://www.ncbi.nlm.nih.gov/). 16S rRNA Sanger sequencing was able to identify isolates 100% to the species level in contrast to conventional methods **(Table 2).**

Conventional method was not able to identify (8/29, 27.5%) isolates from the total isolates, misidentify (10/29, 34.4%) isolates and (7/29, 24.1%) isolates could not be identified to species level. This method was not able to differentiate between pathogenic and nonpathogenic *Pseudomonas* species. In contrast, all colonies were successfully identified with 16S rRNA sequence analysis where isolates were categorized into (18) genera and 22 species. All isolates showed high sequence identity (99%). Sixty-six colonies (categorized into 7 isolates) were presumed to *Pseudomonas* by conventional method. However, 16S rRNA Sanger sequencing indicated 21 out 66 colonies (categorized into 2 isolates) belonged to another genus. *Pseudomonas was* the most common gram-negative genus isolated, followed by *Enterobacter* then *Klebsiella* and *Citrobacter.*

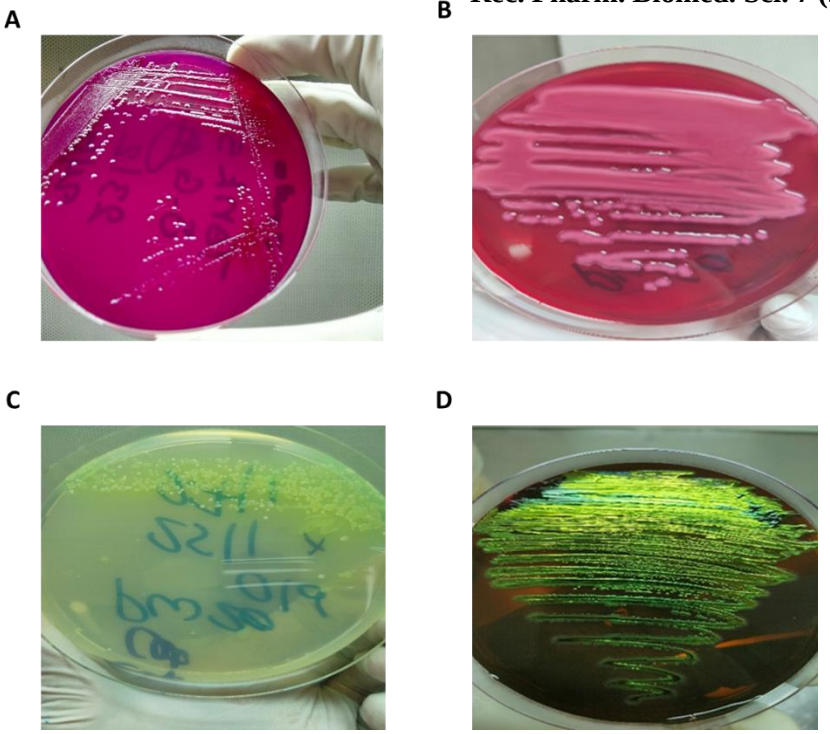

Figure 1. Conventional Identification of cultured plates. A) Pink colonies on Endo agar medium. B) Pink mucoid colonies on MacConkey agar medium. C) Green pigmented colonies on Cetrimide agar. D) Colonies showing green metallic sheen on Eosin Methylene Blue (EMB) agar medium

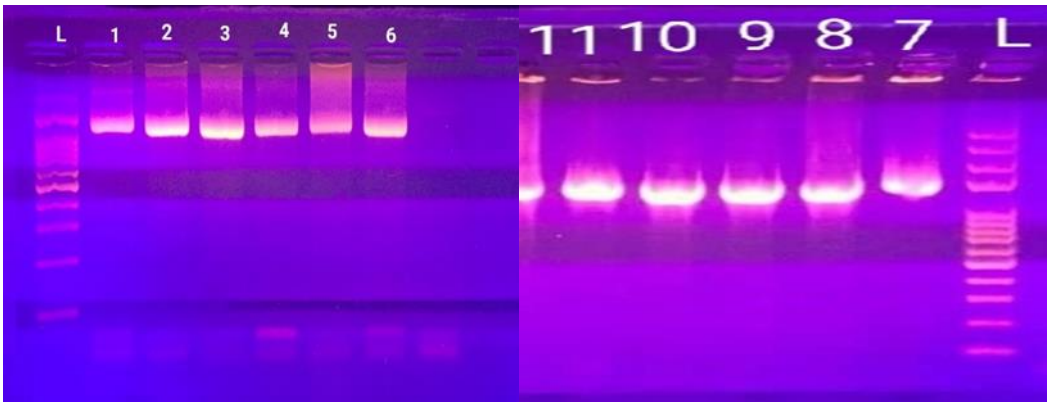

Figure 2. Agarose gel electrophoresis of 16S rRN gene (1500 bp), Lane (L): ladder (100 bp DNA ladder), Lane (1-11): positive results.

Molecular identification using 16S rRNA Sanger method accurately detected contaminants in all pharmaceutical process waters after 24 to 40 hours of incubation, in contrast to 5 to 7 days with the traditional method. An additional sample of highly purified water containing very high stressed microorganisms required 14 days to detect contaminants with the culture-based procedure, whereas molecular identification was able to shorten this time-to-result to few days. *Pseudomonas*, *Enterobacter*, *Citrobacter* and *Klebsiella* species were overwhelmingly which considered the most common pathogenic isolates from the purified water system. Bacterial identification by the conventional methods showed less accuracy compared with molecular identification. These results confirmed the highly accuracy of molecular identification of *pseudomonas* species which considered the most critical pathogens that should not be found in water samples.

Pharmaceutical market seeks for rapid drug release which depends on rapid, accurate identification of pathogenic bacteria in finished products. The aim of the current study was to isolate and identify the bacteria found in the purified water samples collected from pharmaceutical factory. We have shown that genotypic methods based on 16S rDNA Sanger sequencing improved the identification of gram-negative bacteria compared to conventional phenotypic methods.

Table 2. Bacterial identification results according to the conventional methods and 16S rRNA Sanger sequencing

Isolates	Colonies			Biochemical tests						Identification	
	Morphology	Gram stain	No. of colonies	Oxidase	Indole	TSI	Citrate	MR	Urease	Conventional (Identification)	16S rRNA Sequencing
1	Circular, Small, Beige, Smooth	G-	18	+	-	-	-	-	-	*Pseudomonas aeruginosa*	*Ralstonia pickettii*
2	Circular, Small, Yellow-Orange, Smooth	G-	12	-	-	-	-	-	-	-	*Chryseobacterium pallidum*
3	Circular, Small,White, Smooth	G-	11	-	-	-	-	-	-	-	*Mitsuaria chitosanitabida*
4	Circular, Large Yellow, Smooth	G-	10	-	-	+	+	+	-	*Enterobacter sp.*	*Enterobacter cloacae*
5	Circular Small, Dark Pink	G-	12	+	-	-	-	-	+	-	*Methylobacterium radiotolerans*
6	Circular, Medium, Beige, Smooth	G-	28	+	-	-	+	+	-	*Pseudomonas putida*	*Pseudomonas aeruginosa*
7	Circular, Large Buff, Smooth	G-	17	-	-	+	+	+	-	*Enterobacter sp.*	*Enterobacter hormaechei*
8	Circular, Medium, Beige, Smooth	G-	4	+	-	-	+	+	-	*Pseudomonas sp.*	*Pseudomonas putida*
9	Circular, Large Buff, Smooth	G-	3	+	+	-	-	+	+	*Burkholderia cepacia*	*Burkholderia cepacia*
10	Circular, Translucent, Medium, Smooth	G-	10	-	-	-	+	+	-	*Citrobacter freundii*	*Citrobacter freundii*
11	Circular, Large ,Buff, Smooth	G-	1	+	+	-	-	+	+	-	*Burkholderia vietnamiensis*
12	Circular, Medium, Yellow, Smooth	G-	4	-	+	-	-	+	+	*Acinetobacter lwoffii*	*Stenotrophomonas maltophilia*
13	Circular, Translucent, Medium, Smooth	G-	11	-	-	-	+	+	-	*Citrobacter freundii*	*Citrobacter freundii*
14	Circular, Medium, Yellow, Smooth	G-	2	-	+	-	-	+	+	*Stenotrophomonas maltophilia*	*Acinetobacter lwoffii*

15	Circular Creamy White, Medium ,Smooth	G-	4	+	-	-	+	+	-	*Pseudomonas sp.*	*Pseudomonas putida*
16	Circular Creamy white, Large, Shiny, Mucoid	G-	13	-	-	-	+	-	+	*Kluyvera intermedia*	*Klebsiella pneumoniae*
17	Circular Creamy white, Large, Shiny, Mucoid	G-	11	-	-	-	+	-	+	*Klebsiella pneumonia spp*	*Klebsiella aerogenes*
18	Circular Creamy White, Medium ,Smooth	G-	7	+	-	-	+	+	-	*Pseudomonas sp.*	*Pseudomonas fluorescens*
19	Circular, Medium, Bright Pink, Smooth	G-	6	-	+	-	-	+	+	*Stenotrophomonas maltophilia*	*Shewanella putrefaciens*
20	Circular, Buff , Large, Shiny, Mucoid	G-	14	-	-	-	+	-	+	*Klebsiella pneumonia spp*	*Kluyvera intermedia*
21	Circular, Medium, Beige, Smooth	G-	1	+	-	-	-	-	-	-	*Aeromonas hydrophila*
22	Circular, Brown, Medium, Smooth	G-	1	-	-	-	-	-	-	-	*Erwinia amylovora*
23	Circular, Medium, Red ,Smooth	G-	5	-	+	-	-	-	-	-	*Serratia ureilytica*
24	Circular, Large, Yellow , Smooth	G-	6	-	-	+	+	+	-	*Enterobacter clocae*	*Pantoea agglomerans*
25	Circular, Small, White, Smooth	G-	3	-	-	-	-	-	-	-	*Roseateles depolymerans*
26	Circular, Large, Buff, Smooth	G-	12	-	-	+	+	+	-	*Enterobacter sp.*	*Enterobacter cloacae*
27	Circular, Small, Beige, Smooth	G-	3	+	-	-	-	-	-	*Pseudomonas aeruginosa*	*Ralstonia pickettii*
28	Circular ,Buff, Medium ,Smooth	G-	2	+	-	-	+	+	-	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*
29	Circular ,Buff, Medium ,Smooth	G-	4	+	-	-	+	+	-	*Pseudomonas Sp.*	*Pseudomonas fluorescens*
Total Isolates	**235**										

We found that 16S rRNA gene sequences frequently provide phylogenetically useful information. Signature nucleotides allow classification even if a particular sequence has no match in the database, since otherwise-unrecognizable isolates can be assigned to a phylogenetic branch at the class, family, genus, or subgenus level. We also note that identification of slow-growing or biochemically inert gram-negative bacilli to the species level is difficult and time-consuming by conventional methods **(Gernerer *et al.*, 1991).**

The present study provided evidence suggesting that: Pharmaceutical water harbors a diverse and dynamic microbial community. Many of the bacteria are difficult-to-grow using the conventional methods. Sequencing data done in this study could be used to develop assays for the monitoring of potentially active bacteria in pharmaceutical water systems. Time taken for complete analysis and identification of microorganisms in each sample was between 3-7 days, while the time needed in rapid methods was only 24 hrs. The molecular identification would solve the problem of not identified or misidentified isolates which is a critical problem for the microbiology laboratories in quality control departments.

6. References

Amann, R. I., Ludwig, W., & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological reviews, 59(1), 143-169.

Atashpaz, A., Barzegari, A., & Azarbaijani, R. (2008). General DNA extraction kit. Iranin Patent Office, (48024).

Bartram, J., Cotruvo, J. A., Exner, M., Fricker, C., & Glasmacher, A. (Eds.). (2003). Heterotrophic plate counts and drinking-water safety. IWA publishing.

Bartram, J., Cotruvo, J., Exner, M., Fricker, C., Glasmacher, A., Water, S., & World Health Organization. (2003). Heterotrophic plate counts and drinking-water safety: the significance of HPCs for water quality and human health/J. Bartram.

Bordner R., Winter J. and Scarpino P. (ed). (1978). Microbiological Methods for Monitoring the Environment: Water and Wastes. EPA-600/8-78-017, Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, OH,

Brink, B. (2010). Urease test protocol. Washington, DC: American Society for Microbiology.

Chagnaud, P., Machinis, K., Coutte, L. A., Marecat, A., and Mercenier, A. (2001). Rapid PCR –based procedure to identify lactic acid bacteria: application to six common Lactobacillus species. J. Microbiol. Methods 44, 139–148. doi: 10.1016/S0167-7012(00)00244-X

Chowdhury, S. (2012). Heterotrophic bacteria in drinking water distribution system: a review. Environmental monitoring and assessment, 184(10), 6087-6137.

Clarridge III, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clinical microbiology reviews, 17(4), 840-862.

Cloud JL, Harmsen D, Iwen PC, et al. Comparison of traditional phenotypic identification methods with partial 5' 16S rRNA gene sequencing for species-level identification of nonfermenting Gram-negative bacilli. J Clin Microbiol. 2010;48(4):1442-1444. doi:10.1128/JCM.00169-10

Felske, A., W. M. De Vos, and A. D. L. Akkermans. "Spatial distribution of 16S rRNA levels from uncultured acidobacteria in soil." Letters in applied microbiology 31.2 (2000): 118-122.

Gerner-Smidt P, Tjernberg I, Ursing J. Reliability of phenotypic tests for identification of Acinetobacter species. J Clin Microbiol. 1991; 29:277–282.

Heijs SK, Haese RR, van der Wielen PW, Forney LJ, van Elsas JD. (2007). Use of 16S rRNA gene-based clone libraries to assess microbial communities potentially involved in anaerobic methane oxidation in a Mediterranean cold seep. Microb Ecol. 53(3):384-398. doi:10.1007/s00248-006-9172-3

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., ... & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nature communications, 10(1), 1-11.

Lane, D. J. (1991). “16S/23S rRNA Sequencing,” in Nucleic Acid Techniques in Bacterial Systematic, eds E. Stackebrandt, and M. Goodfellow, (New York, NY: John Wiley and Sons), 115–175.

Lehman, D. (2005). Triple sugar iron agar protocols. Microbe Library.

MacWilliams, M. P. (2009). Citrate test protocol. American Society for Microbiology.

McDevitt, S. (2009). Methyl red and voges-proskauer test protocols. American Society for Microbiology, 8.

Morgan, S.M., O'Sullivan, L., Ross, R.P. and Hill, C. (2002) The design of a tree strain starter system for Cheddar cheese manufacture exploiting bacteriocin-induced starter lysis. Int Dairy J 12, 985– 993.

Nemati, M., Hamidi, A., Dizaj, S. M., Javaherzadeh, V., & Lotfipour, F. (2016). An overview on novel microbial determination methods in pharmaceutical and food quality control. Advanced pharmaceutical bulletin, 6(3), 301.

Noble, R. T., & Weisberg, S. B. (2005). A review of technologies for rapid detection of bacteria in recreational waters. Journal of water and health, 3(4), 381-392.

Ph. Eur. monograph “Water, purified” (0008).

Reasoner, D. J., & Geldreich, E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. Applied and environmental microbiology, 49(1), 1-7.

Reasoner, D., 2004. Heterotrophic plate count methodology in the United States. International Journal of Food Microbiology, 92(3), pp.307-315.

Revetta, R. P., Pemberton, A., Lamendella, R., Iker, B., & Santo Domingo, J. W. (2010). Identification of bacterial populations in drinking water using 16S rRNA-based sequence analyses. Water Research, 44(5), 1353-1360.

Sandle, T., 2016. Microbial identification. Pharmaceutical Microbiology, pp.103-113.

Shields, P., & Cathcart, L. (2010). Oxidase test protocol. American Society for Microbiology, 1-9.

Szewzyk U, Szewzyk R, Manz W, Schleifer KH. Microbiological safety of drinking water. Annu Rev Microbiol. (2000). 54:81-127. doi: 10.1146/annurev.micro.54.1.81. PMID: 11018125.

United States Pharmacopeia (USP) 44 NF 39 Chapter <1231> chapter Water for Pharmaceutical Purposes, (2021). 7742-7778.

WHO good manufacturing practices: water for pharmaceutical use. Annexure -2, https://www.who.int/medicines/ areas/quality_safety/quality_assurance/TRS986annex2.pdf.