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The 1st Cirebon International Health Symposium: Faculty of Medicine, Universitas Swadaya Gunung Jati *Update on Non-Communicable Diseases: Global Perspective on Health Challenges and Innovation*

Examination of *Coliform* and *Escherichia coli* Contamination in Refilled Drinking Water in Cirebon Regency, Indonesia

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DOI: 10.35898/ghmj-82s1224

ABSTRACT

Background: Refillable drinking water is a commonly used source of water for the public. Contamination of this water can lead to various diseases, including waterborne diarrhea. *Coliform* bacteria and *Escherichia coli* are indicators of faecal contamination and can cause waterborne diseases. This study aims to determine whether *Coliform* and *Escherichia coli* are present in refillable drinking water in Cirebon Regency.

Aims: To identify the presence of *Coliform* and *Escherichia coli* bacteria in refillable drinking water in Cirebon Regency.

Methods: This descriptive study employed purposive sampling to collect six samples of refillable drinking water from six different depots located in Cirebon Regency. Each sample was tested four times using various laboratory tests, including Nutrient Agar, Mac Conkey Agar, Gram Staining, IMVIC, and TSIA.

Results: All samples showed the presence of *Coliform* (100%), as shown by the Mac Conkey Agar test indicating lactose fermentation and Gramme Staining revealing rod-shaped bacteria. However, *Escherichia coli* was not found (0%) in any of the samples. The results of the Mac Conkey Agar test as a lactose fermenter, Gram Staining showing rod-shaped bacteria, and IMVIC tests showed the following profile: Indole +, MR +, VP -, Citrate -, and TSIA: A/A, H_2S -, Gas +. While *Escherichia coli* was absent, the detection of other *Coliforms* suggests persistent hygiene issues; *Citrobacter freundii* and *Klebsiella* were found to be *Coliform* bacteria.

Conclusion: No *Escherichia coli* were detected in any of the samples, but *Coliform* bacteria were found in 6 out of 6 samples (100%). The maintenance of refillable drinking water depots is crucial for public health. Owners must frequently clean and disinfect the reservoirs to prevent contamination. Additionally, authorities are responsible for regularly monitoring the hygiene and sanitation of these depots.

Keywords: *Coliform; Escherichia coli; Refillable drinking water.*

Received: 03 July 2025 **Reviewed:** 15 July 2025 **Revised:** 25 July 2025 **Accepted:** 15 August 2025.

1. Introduction

Refill water depots are companies that process raw water into drinking water and then market it directly to consumers. The price of refill drinking water is more affordable compared to bottled drinking water, so refill water is the choice compared to branded drinking water (Arumsari et al., 2021). The Central Statistics Agency stated that 40.64% of Indonesian households use refill water as their source of drinking water (Muhammad, 2023). Water can be a medium for transmitting disease to humans if it has been polluted and contaminated by pathogenic microorganisms such as bacteria (Rophi, 2022).

Detection of microbiological contamination such as *Escherichia coli* and *Coliform* bacteria is the key to drinking water quality. Drinking water quality is an important aspect of health. Water contaminated with *Coliform* bacteria indicates potential contamination, while *Escherichia coli* is a type of bacteria included in the *Coliform* group that shows more serious faecal contamination (Saputri et al., 2020). Water that has been contaminated with *Escherichia coli* and *Coliform* bacteria that are transmitted through water and can cause public health problems, one of which is diarrhea. Waterborne disease is a disease transmitted through drinking water that is directly contaminated with pathogenic microorganisms or substances in the air. Most waterborne diseases are characterised by diarrhea involving excessive expulsion of large amounts of water, often resulting in dehydration and possible death (Khairunnida et al., 2020). *Escherichia coli* is a rod-shaped bacterium that is Gram negative, facultative anaerobic, does not form spores, and is a natural flora in the mammalian intestine. *Escherichia coli* can cause complications such as dehydration, chronic diarrhea, and haemolytic uraemic syndrome, which are often suffered by children under 5 years of age and over 60 years of age. *Escherichia coli* can be found in soil, vegetables, undercooked meat, and air (Mueller et al., 2023).

Based on WHO data from 2015, diarrhea causes around 688 million people to become sick, and 499,000 deaths worldwide occur in children under 5 years of age (KEMENKES, 2017). Based on the 2019 Indonesian Health Profile, the number of diarrhea sufferers in Indonesia is 2,549 people, and the Case Fatality Rate (CFR) is 1.14% (Nariswari et al., 2023). The prevalence of diarrhea in West Java Province based on the 2018 Riskesdas was recorded at 1,287 (10.40%) children with diarrhea in the age group under 1 year, 5,312 (13.43%) children with diarrhea in the age group 1-4 years, 12,806 (6.98%) children with diarrhea in the age group 5-14 years, and 12,409 (7.24%) children with diarrhea in the age group 15-24 years. Meanwhile, in Cirebon City, the incidence of diarrhea in 2018 was 789 cases of diarrhea. According to the Decree of the Minister of Health of the Republic of Indonesia Nomor. 492/Menkes/Per/IV/2010, the microbiological parameters that need to be checked in drinking water are the absence of detection of *Coliform* and *Escherichia coli* bacteria in 100 ml of water samples. There is no current research on the *Coliform* and *Escherichia coli* identification test using nutrient agar, Mac Conkey, Gram staining, IMVIC, and TSIA media tests in Cirebon, West Java. This study was conducted to identify the presence of *Coliform and Escherichia coli* bacteria by using nutrient agar, Mac Conkey, Gram staining, IMVIC, and TSIA media tests from refillable drinking water in Cirebon regency.

2. Methods

This study uses a descriptive method with nutrient agar, Mac Conkey agar, gramme staining, IMVIC, and TSIA. In this study, 6 samples were obtained from refill drinking water depots located in Cirebon Regency using the Purposive Sampling technique that considers cleanliness and sanitation. Based on urban balance, six depots are chosen, and they are then randomly assigned based on predetermined criteria, like the requirement that the water source be from the regional drinking water company / Perusahaan Daerah Air Minum (PDAM) source. The inclusion criteria in this study were refill drinking water depots located in Cirebon Regency, and the exclusion criteria were refill drinking water whose owners refused to be sampled.

Glass bottles that have already been sanitised in an autoclave set to 130 degrees Celsius are used to collect water samples. The container is first sanitised before the water is collected, and then it is carried to the testing location in a sterile box that has already been sterilised. Using a micropipette during the test in the BioSafety Cabinet Samples are taken in previously sanitised petri dishes and are cultured in an incubator to prevent contamination. The test standard based on the Decree of the Minister of Health of the Republic of Indonesia

Nomor 492/Menkes/Per/IV/2010 states that the microbiological parameters that must be examined in drinking water include the absence of *Escherichia coli* and *Coliform* bacteria in 100 milliliters of water samples. This study has received scientific approval and ethical approval issued by the Ethics Commission of the Faculty of Medicine, Swadaya Gunung Jati University, Cirebon No.9/EC/FKUGJ/IV/2024.

3. Results

Shown in Table 1, out of all the samples that used Nutrient Agar Media to count the number of colonies, sample 1 had the highest number of bacteria with total number of bacteria 2.533 CFU/ml. The lowest were found in the sample 6 with total number of bacteria 73 CFU/ml. According to Table 2, 6/6 samples cultivated on Mac Conkey media were lactose fermenter. Table 3 indicates that 6/6 samples that underwent Gram staining contained Gram negative. Based on Table 4, the results show that 6/6 samples tested for IMVIC contained characteristics of Coliform bacteria and no characteristics of *Escherichia coli* bacteria were found.

Table 1. Nutrient Agar Media Test Results

Samples	Repetition	Number of colonies	Total Number of Bacteria
1	One	362 CFU/100 ml^2	2.533 CFU/ml
	Two	610 CFU/100 ml^2	
	Three	962 CFU/100 ml^2	
	Four	598 CFU/100 ml^2	
2	One	57 CFU/100 ml^2	272 CFU/ml
	Two	56 CFU/100 ml^2	
	Three	71 CFU/100 ml^2	
	Four	88 CFU/100 ml^2	
3	One	11 CFU/100 ml^2	216 CFU/ml
	Two	26 CFU/100 ml^2	
	Three	116 CFU/100 ml^2	
	Four	63 CFU/100 ml^2	
4	One	320 CFU/100 ml^2	694 CFU/ml
	Two	135 CFU/100 ml^2	
	Three	61 CFU/100 ml2	
	Four	178 CFU/100 ml^2	
5	One	314 CFU/100 ml^2	1556 CFU/ml
	Two	390 CFU/100 ml^2	
	Three	468 CFU/100 ml^2	
	Four	384 CFU/100 ml^2	
6	One	34 CFU/100 ml^2	73 CFU/ml
	Two	4 CFU/100 ml^2	
	Three	22 CFU/100 ml^2	
	Four	13 CFU/100 ml^2	

Table 2. Mac Conkey Media Test Results

No Sample	Repetition	Lactose Fermenter	Non Lactose Fermenter
1	One	+	-
	Two	+	-
	Three	+	-
	Four	+	-
2	One	+	-
	Two	+	-
	Three	+	-
	Four	+	-
3	One	-	+
	Two	-	+
	Three	-	+
	Four	+	-
4	One	+	-
	Two	+	-
	Three	+	-
	Four	+	-
5	One	+	-
	Two	+	-
	Three	+	-
	Four	+	-
6	One	+	-
	Two	+	-
	Three	-	+
	Four	-	+

Table 3. Gram Staining Results

No Sample	Repetition	Result
1	One	Gram negative
	Two	Gram negative
	Three	Gram negative
	Four	Gram negative
2	One	Gram negative
	Two	Gram negative
	Three	Gram negative
	Four	Gram negative
3	One	Are not done
	Two	Are not done
	Three	Are not done
	Four	Gram negative
4	One	Gram negative
	Two	Gram negative
	Three	Gram negative
	Four	Gram negative
5	One	Gram negative
	Two	Gram negative
	Three	Gram negative
	Four	Gram negative
6	One	Gram negative
	Two	Gram negative
	Three	Gram negative
	Four	Gram negative

Table 4. IMVIC Media Test Results

No Sample	Repetition	Description			
		Indol	**Methyl Red**	**Voges Proskauer**	**Citrate**
1	One	-	-	+	+
	Two	-	-	+	+
	Three	-	-	+	+
	Four	-	-	+	+
2	One	-	-	-	+
	Two	-	-	+	
	Three	-	-	+	-
	Four	-	-	+	+
3	One	Are not done	Are not done	Are not done	Are not done
	Two	Are not done	Are not done	Are not done	Are not done
	Three	Are not done	Are not done	Are not done	Are not done
	Four	-	+	+	-
4	One	-	+	+	-
	Two	-	-	+	+
	Three	-	-	+	+
	Four	-	-	+	+
5	One	+	-	-	-
	Two	-	-	+	-
	Three	+	-	-	-
	Four	-	-	+	+
6	One	-	-	+	+
	Two	-	-	+	+
	Three	Are not done	Are not done	Are not done	Are not done
	Four	Are not done	Are not done	Are not done	Are not done

Out of all the samples that used Nutrient Agar Media to count the number of colonies, Sample 1 had the most bacteria, with a total of 2.533 CFU/ml. The lowest concentrations were found in sample 6, with a total of 73 CFU/mL. Six out of six samples grown on Mac Conkey media fermented lactose. After undergoing Gram staining, Gram negative was found in six of the six samples. The results show that none of the six samples tested for IMVIC had features of *Escherichia coli,* but they did show signs of *Coliform bacteria.* A number of samples were not gram-stained following the Mac Conkey test since the findings indicated colours other than red, which is not indicative of Gram-negative bacteria. Several samples were not tested using the IMVIC test since they were found to be Gram-positive bacteria, whereas this study only evaluated Gram-negative bacteria.

Table 5. TSIA Test Results

No Sample	Repetition	Description
1	One	K/K
	Two	K/K
	Three	K/K
	Four	K/K
2	One	K/K
	Two	A/K
	Three	A/K, Gas
	Four	K/A
3	One	Are not done
	Two	Are not done
	Three	Are not done
	Four	K/A
4	One	A/K
	Two	K/K
	Three	A/A
	Four	A/K
5	One	A/A
	Two	A/A, H_2S
	Three	A/A
	Four	A/K
6	One	A/K
	Two	K/K
	Three	Are not done
	Four	Are not done

Observation:
TSIA : Triple Sugar Iron Agar
K/K: Red slant and red butt (Red/Red or Alkaline (K)/ Alkaline (K)
A/K : Red slant and red butt (Red/Red or Alkaline (K)/ Alkaline (K)
K/A : Red slant and red butt (Red/Red or Alkaline (K)/ Alkaline (K)
A/A : Red slant and red butt (Red/Red or Alkaline (K)/ Alkaline (K)
H_2S : Hydrogen sulfide

Table 6. Microbiological Test Results of Refill Drinking Water

Sample	Repetition	MCA	Gram stain	CFU/ml[2]	IMVIC	TSIA	Identification	*Coliform*	*Escherichia coli*
1	One	+	-	362	--++	K/K	*Citrobacter freundii*	+	-
	Two	+	-	610	--++	K/K	*Citrobacter freundii*	+	-
	Three	+	-	962	--++	K/K	*Citrobacter freundii*	+	-
	Four	+	-	598	--++	K/K	*Citrobacter freundii*	+	-
2	One	+	-	57	---+	K/K	*Citrobacter freundii*	+	-
	Two	+	-	56	--+-	A/K	*Citrobacter koseri*	+	-
	Three	+	-	71	--+-	A/K, Gas	*klebsiella sp.*	+	-
	Four	+	-	88	--++	K/A	*Enterobakter sp.*	+	-
3	One	-	N.A	11	N.A	N.A	N.A	N.A	N.A
	Two	-	N.A	26	N.A	N.A	N.A	N.A	N.A
	Three	-	N.A	116	N.A	N.A	N.A	N.A	N.A
	Four	+	-	63	-++-	K/A	*Klebsiella aerogenes*	+	-
4	One	+	-	320	--++	A/K	*Pseudomonas sp.*	+	-
	Two	+	-	135	--++	K/K	*Pseudomonas sp.*	+	-
	Three	+	-	61	--++	A/A	*Enterobakter sp.*	+	-
	Four	+	-	178	--++	A/K	*Pseudomonas sp*	+	-
5	One	+	-	314	+---	A/A	*Klebsiella oxytoca*	+	+
	Two	+	-	390	--+-	A/A, H2S	*Citrobacter freundii*	+	-
	Three	+	-	468	+---	A/A	*Morganella sp.*	+	-
	Four	+	-	384	--++	A/K	*Enterobakter sp.*	+	-
6	One	+	-	34	--++	A/K	*Enterobakter sp.*	+	-
	Two	+	-	4	--++	K/K	*Enterobakter sp.*	+	-
	Three	-	N.A	22	N.A	N.A	N.A	N.A	N.A
	Four	-	N.A	13	N.A	N.A	N.A	N.A	N.A

Observation:
MCA : Mac Conkey Agar
NA: Nutrient Agar
IMVIC: Indol, Methyl Red, Voges Proskauer, Citrate
TSIA : Triple Sugar Iron Agar
K/K: Red slant and red butt (Red/Red or Alkaline (K)/ Alkaline (K)
A/K : Red slant and red butt (Red/Red or Alkaline (K)/ Alkaline (K)
K/A : Red slant and red butt (Red/Red or Alkaline (K)/ Alkaline (K)
A/A : Red slant and red butt (Red/Red or Alkaline (K)/ Alkaline (K)
H_2S : Hydrogen sulfida

4. Discussion

The results of the study that has been conducted on the *Escherichia coli* Content Test in Refill Drinking Water in Kedawung District found that 6 samples did not contain *Escherichia coli,* and there were *Coliform* bacteria in the refill drinking water. *Coliform* bacteria are classified as pathogenic bacteria. The presence of these bacteria determines whether the water or sample is contaminated with pathogenic bacteria or not. The presence of these bacteria is usually caused by contamination, and most *Coliform* bacteria reside in the intestines of humans and animals. This study used the Nutrient Agar test, Mac Conkey, Gram Staining, IMVIC and TSIA biochemical tests. The identification test results for 6 samples planted on Mac Conkey Agar media showed that all the bacteria that grew were lactose fermenters. Mac Conkey agar can distinguish Gram-negative bacteria based on their lactose metabolism and is a selective medium that only grows Gram-negative bacteria. Then, Gram-negative bacteria that

ferment lactose will form pink colonies, while bacteria that do not ferment lactose will form white colonies. Bacteria that grow will differ in Mac Conkey Agar media based on the speed and ability of its lactose fermentation (Jung, et al., 2024).

Nutrient Agar media contain meat extract and peptone for the growth of most bacteria and are a universal medium for bacterial growth. 13 The study counted bacteria using Colony Forming Unit (CFU), which measures how many bacterial colonies are present in each gramme or milliliter of the sample, based on the number of plates, the dilution factor, and the volume used. The pour plate method has the advantage of being able to count bacteria that are worthy of being counted, excluding dead bacteria. Additionally, this method can grow both Gram-negative and Gram-positive bacteria, such as *Escherichia coli* and Staphylococcus aureus, and allows these bacteria to spread throughout the media (Azizah et al., 2020).

Gram-negative bacteria produce a red colour because it is related to the content of Mac Conkey media, such as Lactose, neutral red (pH indicator), bile salts, and crystal violet. The growth of Gram-positive bacteria is inhibited by the content of Crystal violets and bile salts. Microorganisms that ferment lactose will produce organic acids, especially lactic acid, which will lower the pH. Neutral red is a pH indicator that changes from pale white to bright red/pink when the pH drops below 6.8. The six samples were bacilli or rods, so they were in the Gram-negative bacteria group. Bacteria can be classified as Gram-negative because after being washed with alcohol, the bacteria lost the crystal violet dye and were then stained using safranin, a second red dye. Alcohol will release the dye in some bacteria but remain in other bacteria. The cell wall in bacteria will affect the colour difference in Gram staining. The cell wall of Gram-negative bacteria has three layers; the outer layer is made of lipopolysaccharide, which gets washed away by alcohol when stained with safranin, making it turn red. The use of Lugol's iodine creates a bond between crystal violet and iodine, which enhances the staining of bacteria. Ethanol creates holes in Gram-negative bacteria, which have many layers of fat that dissolve in ethanol, allowing the crystal violet iodine complex to stay attached to the cell wall; as a result, Gram-negative cells become clear. Therefore, the violet crystal colour cannot stick to gram-negative bacteria. This condition also causes Gram-negative bacteria to be able to bind the red colour of safranin (Amin et al., 2023).

The IMVIC test is a biochemical test consisting of indole, methyl red, voges proskauer and citrate to distinguish the type of bacteria based on the differences in the genus and species of certain bacteria. In the research that has been done, there is one positive sample. Biochemical tests are carried out to determine the limited morphology of bacteria such as bacilli, cocci and spirals, so biochemical tests are needed to determine the nature of bacteria by seeing bacteria reacting to chemical compounds. To determine the nature of bacteria, it is necessary to add different reagents (Umarudin et al., 2020).

Positive indole test is seen from the formation of a red ring and means that the bacteria have the ability to form indole from the breakdown of the amino acid tryptophan after adding Kovac's reagent (Putri et al., 2023). Positive methyl red test is seen from the change in colour of the media to red after being dripped using methyl red reagent and means that the bacteria can produce acid and have the ability to oxidise glucose. This test is positive for Escherichia coli. 18 The Voges-Proskauer test checks if bacteria can make acetyl methyl carbinol (acetoin) by breaking down glucose into acid. If the colour changes to red after adding alpha-naphthol and KOH reagents, it indicates that the bacteria can form acetoin; however, *Escherichia coli* does not produce acetoin, resulting in a negative test result. The citrate test functions to see if bacteria can use citrate as a carbon source (Umarudin et al., 2020).

TSIA is a test that checks for the production of glucose, lactose, sucrose, and gas, which are then fully oxidised to create carbon dioxide and hydrogen gas with the help of the format hydrogenase enzyme. Accumulation of gas under the tube occurs due to the chemical process of hydrogen gas that is not dissolved in the medium (Apriani et al., 2014). In the TSIA test, the bottom and slant are yellow, indicating that there is a change in the acidic atmosphere in the butt and slant. Meanwhile, if the butt is red and the slant shows a yellow colour, it can be concluded that the bacteria only ferment glucose (Apriyanthi et al., 2022).

Coliform bacteria and *Escherichia coli* serve as indicators for assessing the quality of drinking water. The presence of *Coliform* bacteria in drinking water at refill drinking water depots indicates poor drinking water quality. The cause of drinking water sources being contaminated by *Coliform* bacteria is leachate from septic

tanks; the source of groundwater is influenced by the distance from the septic tank to the well. In addition, if the raw water is not optimally processed, for example, in drinking water reservoirs, ultraviolet radiation is not used in disinfection. In addition, the maintenance filters for all refill water tanks are inadequate; this issue is likely due to filters that have been used for a long time. Other factors that cause refill water in Cirebon Regency to contain *Coliform bacteria* include the filler water reservoir not being kept clean and how long the raw water is stored in the reservoir tank, thus affecting the quality of the raw water source. The refill water reservoir's placement beside the road, the unhygienic behaviour of workers, and the lack of regular inspections for drinking water are all contributing factors (Putri et al., 2023). This evidence is in line with the research conducted by Rezkina K and Roslina A with the title Comparison of Growth of *Escherichia coli* and *Salmonella sp*. On the first and second days at the Refill Drinking Water Depot, the results were obtained that there was no growth of *Escherichia coli* and *Salmonella sp.* on drinking water obtained from the depot. In the study, *Escherichia coli* bacteria were not found in refillable drinking water because sampling was conducted only once and did not consider the length of storage at the depot; subsequently, bacteria were detected using Nutrient agar media, Mac Conkey agar, and Gramme staining, followed by confirmatory tests using IMVIC and TSIA. (Rezkina et al., 2024). The detection of other *Coliform* s suggests persistent hygiene issues. Owners of refillable drinking water depots must frequently clean and disinfect the reservoir, and authorities are required to regularly monitor the hygiene and sanitation of drinking water depots.

5. Conclusion

Coliform bacteria were found in refilled drinking water in Cirebon Regency, and no *Escherichia coli* bacteria were found in refilled drinking water in Cirebon Regency. While *Escherichia coli* was absent, the detection of other *Coliform* s suggests persistent hygiene issues. Owners of refillable drinking water depots must frequently clean and disinfect the reservoir, and authorities are required to regularly monitor the hygiene and sanitation of drinking water depots. The limitation of this study is the small sample size, which is due to several depot owners refusing to allow their refillable drinking water to be used as a research sample, along with the limited geographical spread. The suggestion for further research is to obtain a larger sample that accurately represents the distribution of refillable drinking water depots across the Cirebon district, and to further investigate the impact of piping inlets on the chemical and microbiological quality of refilled drinking water.

Conflict of Interest

There is no conflict of interest.

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Cite this article as:

Shonia, A. N., Fitriani, H., & Indrakusuma, M. E. (2025). Examination of Coliform and Escherichia coli Contamination in Refilled Drinking Water in Cirebon Regency, Indonesia. *GHMJ (Global Health Management Journal)*, *8*(2s), 275–284. https://doi.org/10.35898/ghmj-82s1224