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(RESEARCH ARTICLE)

Bacteriological analysis of hospital sewage of district Hospital Ratnagiri

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Abstract

Hospital sewage is a waste drained out from the various clinical facilities of the hospital. The hospital sewage contains pathogens which pose severe public health threat. Most hospitals lack sewage treatment facilities and discharge the sewage untreated directly in to the public sewer. The study was aimed to analyze the hospital sewage of District hospital Ratnagiri for Coliform count by MPN and serial plate dilution method, and identification of the Bacterial isolates by conventional method from the sewage. Physico-chemical parameters like Temperature, pH, TDS, COD, BOD5, samples were determined which affect the bacterial flora of the sewage. Presumptive and Confirmatory MPN test and Serial plate dilution was performed. Coliform were isolated and identified using Gram stain, Colony characteristics and Biochemical characteristics. Temperature was 28(+1.22)°c, pH 6.52(+0.10), TDS 251(+1.41) mg/l, COD 96.9(+0.82) mg/l and BOD 69.5 (+0.58) mg/l. TDS was higher, COD and BOD values were less as compare to the permissible limits. Presumptive MPN was >1.8 x 103, Confirmatory MPN was 3.3x101 per 100 ml and coliform count by serial dilution was 6x106 CFU/ml on Blood agar and 2x106 CFU/ml on MacConkey's agar which indicated higher counts of coliform in the sewage. Common bacteria like Escherichia coli, Pseudomonas aeruginosa, *Klebsiella peumonae, Chromobacter violaceum* and *Bacillus subtilis* were isolated. The study reveals presence of significant coliform load in the hospital sewage which poses risk of infection to the community.

Keywords: Hospital sewage; Static Bioassay; Most Probable Number; Coliform count; Serial dilution

1. Introduction

With increase in human population, there is also a proportional increase in health care facilities to serve the increasing health care demands. The hospital activities generate a considerable amount of Health care waste which is referred as "Biomedical Waste" during its operation. Biomedical waste includes Solid waste & Liquid waste. Many Biomedical waste treatment plants provide facility for management of solid wastes, but most of the liquid waste is flushed in to hospital drainage system which forms a significant proportion of hospital sewage. The hospital sewage comprises of waste waters drained out from washing & sterilization units, Laboratories, Treatment & Surgical areas, Radiotherapy and Radiology units, Laundry and Kitchen waste etc.

The composition of Hospital sewage is more complex as compared to the municipal sewage. It is well established that the hospital sewage contains pathogens, human products (tissues, fluids, and excreta), pharmaceutical substances, chemicals, heavy metals and radioactive wastes which are infectious and genotoxic.[1,2,3] The hospital waste waters mix up with the streams, rivers, lakes and ocean, waters from which are utilized for recreation, drinking, agriculture and industrial purpose.[4]

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The discharge of untreated hospital sewage causes environmental problems in the same way as that of urban effluent. Hospital sewage contains high coliform count more than the international permissible limits recommended by WHO as compared to municipal or domestic sewage.[5] The major impact of hospital sewage is due to its heavy content of variety of pathogenic organisms including viruses, bacteria, fungi, protozoa and helminthes ova. The hospital waste water entering the local water bodies poses threat to its consumers like animals and humans. Also the pathogenic as well as chemical contents have potential to cause severe impact on water inhabiting organisms like fishes, molluscs, other aquatic animals and terrestrial animals and birds feeding on them including humans.

There are number of studies on hospital waste focusing mainly on the physico-chemical characteristics and coliform counts. Some studies are found to be aimed at the identification of microorganisms. There is lack of similar studies in Konkan area of Ratnagiri district which is popular for its biodiversity and nature. District Hospital Ratnagiri is a major tertiary care hospital in Ratnagiri district with large number of patients visiting here for treatment of variety of ailments. The hospital lacks sewage treatment plant and the hospital effluent is drained out into the municipal drainage system directly. Our study aimed to find out the Coliform count, Coliform load in the sewage sample and identification of the coliform isolated in the hospital sewage of the District Hospital Ratnagiri.

2. Material and methods

Hospital sewage sample of about 500 ml was collected in accordance with aseptic handling techniques using pre-sterilized plastic sampling containers from the common outlet of District Hospital Ratnagiri. Sample was particularly collected during morning between 09:00 am to 10:00 am from January 2017 to December 2018.

Figure 1 Common drainage site of sewage collection at District hospital Ratnagiri

Ambient Temperature and Sewage temperature was recorded using a mercury thermometer. pH was determined using pH meter (Rolex scientific engineers sr. no. Mj-2015/07/333, model no.10).

2.1. Presumptive Coliform count

Presumptive Coliform count was determined using Most Probable Number (MPN) technique.[6] The procedure for MPN was performed as follows-

- The collected sewage sample was mixed thoroughly by inverting the bottle several times
- The cap of the bottle was removed and the mouth of the bottle was flamed.
- 15 tubes were arranged in three rows (R1, R2, R3) with 5 tubes in each row
- 10 ml sample was inoculated in first row R1 of five tubes each containing 10 ml double strength MacConkey's broth with inverted Durham's tube
- 1 ml sample was inoculated in second row R2 of five tubes each containing 5 ml single strength MacConkey's broth

- ml sample was inoculated in third row R3 of five tubes each containing 5 ml single strength MacConkey's broth
- All inoculated tubes were incubated at 37°c for 24 hours.
- Results for coliform count were noted for untreated hospital sewage as per the standard MPN table.

The following table summarizes the procedure for five tube MPN method

Table 1 Addition of sewage sample in tubes with MacConkey's broth for five tube MPN Method

Row	MacConkey's broth strength	MacConkey's broth (ml) Tube (T)	Sewage Sample
R1	Double strength (ds)	T_{ds1}- 10 ml	10 ml
		T_{ds2}- 10 ml	10 ml
		T_{ds3}- 10 ml	10 ml
		T_{ds4}- 10 ml	10 ml
		T_{ds5}- 10 ml	10 ml
R2	Single strength (ss)	T_{ss1}- 10 ml	1 ml
		T_{ss2}- 10 ml	1 ml
		T_{ss3}- 10 ml	1 ml
		T_{ss4}- 10 ml	1 ml
		T_{ss5}- 10 ml	1 ml
R3	Single strength (ss)	T_{ss1}- 10 ml	0.1 ml
		T_{ss2}- 10 ml	0.1 ml
		T_{ss3}- 10 ml	0.1 ml
		T_{ss4}- 10 ml	0.1 ml
		T_{ss5}- 10 ml	0.1 ml

2.2. Confirmatory test

Further confirmatory test was carried out by transferring one to two drops using sterile nichrome wire loop from positive presumptive tubes into fresh tubes with 10 ml of confirmatory broth with inverted Durham's tube. The tubes were incubated at 44°c for 24 hours. The tubes were observed for production of acid and gas at the end of 24 hours.

2.3. Completed test

The loop full of aliquot from the positive confirmatory tube with lowest dilution was inoculated on Eosin Methylene blue (EMB) agar by four quadrant method. The plate was incubated at 37°c for 24 hours. The plate was observed for growth after the end of 24 hours.

2.4. Serial Dilution method for bacterial count

The sewage sample was processed for bacteriological examination by using serial dilution method.[7]

Six tubes labeled as T1 to T6 were arranged in a row and 9 ml of sterile 0.85% saline was added to each tube. Sample was used undiluted for the procedure. 1 ml of undiluted sewage sample was added to first tube and mixed well. Next 1 ml of content from tube 1 was transferred to tube 2. Serial transfer of 1 ml content from previous tube to the next tube was carried up to the last tube and 1 ml of content from the 6^{th} tube was discarded.

0.1 ml of diluted sample from each of the 6 tubes was inoculated on separate Blood agar & MacConkey's agar plates by spread plate technique with the help of glass spreader. All plates were incubated at 37°c for 24 hours. Colony count was

determined by counting the colony forming units using colony counter (*Bio enterprises sr. no. 483, Model no. 887*). The bacterial colony count was derived by considering following parameters.

The countable plate was considered with colony count between 30 to 300 colonies. More than 300 colonies are too numerous to count while colonies less than 30 are too less to count. The number of colonies is the number of Colony Forming Units (CFU) which represents the number of bacteria per ml.

The sample dilution factor (SDF) is the dilution of sewage sample using sterile distilled water. The sample was used undiluted, so the sample dilution factor is 1/1.

The Individual Tube dilution factor (ITDF) is the amount of sample added to the individual tube divided by the total volume in the tube after adding the sample. It indicates how much the sample was diluted in each individual tube. In the first tube (T1), 1 ml of sample and 9 ml of sterile water was added, so the ITDF for the tube is calculated to be 1ml/(1ml + 9 ml) = 1/10. The individual tube dilution is 1:10 for sewage sample.

The total series dilution factor (TSDF) is the multiplication of individual ITDF up to the dilution with countable plate i.e. ITDF for T1x ITDF for T2x…. upto the tube representing the countable plate.

Plate dilution factor (PDF) is the volume of sample inoculated on the countable plate and from which colony count is represented as colony forming unit per ml.

2.5. Identification of bacterial isolates

Colonies with different characteristics on Blood agar and MacConkey's agar from serial dilution method were picked up with sterile nichrome wire loop and sub cultured on new plates of Blood agar and MacConkey's agar. The isolated colonies were further identified by colony characteristics on Blood agar and MacConkey's agar, Gram's staining and by conventional Biochemical reactions like Voges proskauer, Indole, Methyl red, Oxidase, Citrate utilization, catalase, urease, Gelatinase, Motility, TSI and fermentation of sugars like Glucose, Sucrose and Lactose.[8]

3. Results

3.1. Physico-chemical analysis

The physico-chemical analysis of the sewage sample collected from the common drainage outlet of District hospital Ratnagiri was done to determine Temperature, pH, BOD and COD. The findings are presented in the following table.

Table 2 Physico-chemical parameters of sewage samples collected from District hospital, Ratnagiri from January 2017 to December 2018

Parameters	Values	Permissible limits[9]
Temperature (°c)	28 (±1.22)	---
TDS (mg/l)	251 (±1.41)	<600
pH	6.52 (±0.10)	5.5 to 9.0
COD (mg/l)	96.9 (±0.82)	<250
BOD_5 (mg/l)	69.5 (±0.58)	<350

3.2. Presumptive isolation of coliform bacteria

The Presumptive isolation of coliform bacteria was performed using the Most Probable Number (MPN) method using five test tubes with MacConkey's broth (10ml x5, 1mlx5, 0.1mlx5) using variable volumes of Sewage sample as shown in the Table No. 1. The findings are presented in the Table No. 3.

Table 3 Results of presumptive isolation of coliform bacteria using the Most Probable Number (MPN) method

Parameters	R1	R2	R3	MPN index /100 ml
No. of tubes used	5	5	5	>1800
Volume of MacConkey's broth in each tube	10 ml	10 ml	10 ml	
Volume of sewage sample in each tube	10 ml	1 ml	0.1 ml	
No. of tubes showing acid & gas formation	5	5	5	

The observations of MPN were compared with the standard five tube MPN table for untreated sample. The MPN was found to be >1800 coliform per 100 ml. This indicated heavy bacterial load of the sewage sample.

Further confirmatory test carried out by sub culturing the growth from positive presumptive tubes into fresh tubes containing confirmatory broth with inverted Durham's tube and incubated at 44°C for 24 hours were observed for production of acid and gas. The findings are represented in Table No. 4 below.

Table 4 Results of confirmatory test for fecal coliform bacteria using the using the Most Probable Number (MPN)

Parameters	R1	R2	R3	MPN index per 100 ml
No. of tubes used	5	5	5	33
Volume of confirmatory broth in each tube	10 ml	10 ml	10 ml	
Volume of sewage sample in each tube	10 ml	1 ml	0.1 ml	
No. of tubes showing acid & gas formation	5	1	0	

The completed test where sample from the positive tube with smallest sample volume i.e. first tube of the second row (R2) was inoculated on Eosin Methylene Blue (EMB) agar by 4 quadrant method after incubation at 37 °c for 24 hours the plate showed growth of coliform with metallic sheen. This confirmed the presence of the coliform in the sewage sample.

3.3. Bacterial count using serial dilution method

Sample dilution obtained by serially transferring 1 ml of sewage sample in the series of 6 tubes with 9 ml of 0.85% saline and subsequent inoculation of 0.1 ml aliquot from each tube on the separate blood agar and MacConkey's agar plates, after incubation at 37°c for 24 hours showed the results as represented in the following table.

Table 5 Serial dilution method for bacterial count in sewage sample

Tube no.	Dilution	Blood agar (CFU/ 0.1 ml)	MacConkey's agar (CFU/ 0.1 ml)
1	10^{-1}	>300	>300
2	10^{-2}	>300	>300
3	10^{-3}	>300	>300
4	10^{-4}	60	20
5	10^{-5}	50	0
6	10^{-6}	15	0

The countable plate was found to be sample inoculated from the tube no. 4 with 10^{-4} dilution. As the sample was undiluted, the Sample Dilution Factor (SDF) was 1:1. The Individual tube dilution factor (ITDF) for the sewage sample was 1/10. In all the tubes combined, the Total series dilution factor (TSDF) was calculated to be 1/10000. The Plating Dilution Factor (PDF) was 1/10. Final Dilution factor (FDF) was calculated to be 1/100000. Colony forming unit per ml in the original sewage sample was derived by multiplying colony forming units on countable plate by 1/FDF as shown below.

The countable plate had 60 CFU on Blood agar and 20 CFU on MacConkey's agar, and the FDF was 1/100000.

Therefore on blood agar 60 CFU x 1/1/100000 = 60 CFU x 100000 = 6000000 CFU/ml = 6 x 10^6 in the original sample

On MacConkey's agar 20 CFU x 1/1/100000 = 20 CFU x 100000 = 2000000 CFU/ml = 2 x 10^6 in the original sample

This indicates that there is heavy bacterial load in the original sewage sample

Figure 2 MacConkey's agar plate with Pink (Lactose fermenters), White (Non lactose fermenters) colonies in 10^{-4} serial dilution of sewage sample showing 20 CFU

Figure 3 Colonies on Blood agar in 10^{-4} serial dilution of sewage sample showing 60 CFU

3.4. Identification of bacterial isolates from sewage

The microbial analysis showed five different isolates of which four were Gram negative bacilli and one was Gram positive bacilli with endospores. The colony characteristics, gram nature and biochemical characteristics showed following result represented in table no. 6 below

Table 6 Biochemical Characteristics of the bacterial isolates

Features	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Colony on Blood agar	Non hemolytic, 2mm, grey moist colonies	Non hemolytic, 2mm, grey mucoid colonies	B-hemolytic, 3 mm, Black colonies	B-hemolytic, 3mm, smooth colonies, grayish black	B- hemolytic, 3 mm, grey colonies with irregular margin
Colony on MacConkey's agar	Pink colonies, circular, low convex, smooth, translucent, colonies	Pink colonies, circular, low convex, smooth, translucent, colonies	Non lactose fermenting colonies,	Non lactose fermenting colonies	No growth
Colony on EMB agar	Green metallic sheen	Pink mucoid colonies	---	---	---
Gram staining	Gram negative bacilli	Gram negative bacilli	Gram negative coccobacillus	Gram negative bacilli	Gram positive bacilli with endospores
Voges Proskauer	Negative	Positive	Negative	Negative	Positive
Methyl Red	Positive	Negative	Negative	Negative	Negative
Indole	Positive	Negative	Negative	Negative	Negative
Motility	Positive	Non motile	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative	Positive	Negative
Citrate utilization	Negative	Positive	Negative	Positive	Positive
Catalase	Positive	Positive	Positive	Positive	Positive
Urease	Negative	Positive	Negative	Negative	Negative
Glucose	Fermented with Gas	Fermented with Gas	Fermented, No gas	Fermented, No gas	Fermented No gas
Sucrose	Fermented with Gas	Fermented with Gas	Not fermented	Not fermented	Fermented no gas
Lactose	Fermented with Gas	Fermented with Gas	Not fermented	Not fermented	Not fermented
TSI	A/A with Gas, No H2S	A/A with Gas, No H2S	K/A No gas, No H2s	K/K No gas & No H2S	K/A, No gas & No H2S
Gelatinase	Positive	Positive	Positive	Positive	Positive
Identification	*Escherichia coli*	*Klebsiella pneumonae*	*Chromobacter violaceum*	*Pseudomonas aeruginosa*	*Bacillus subtilis*

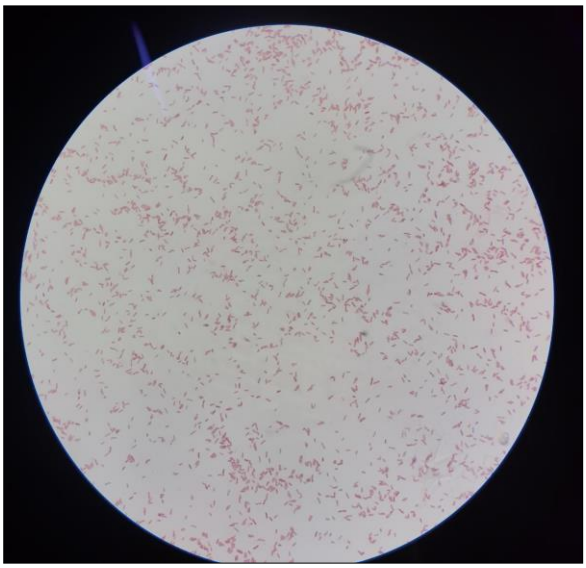

Figure 4 Microscopy of isolates: Gram negative bacilli under oil immersion (x100)

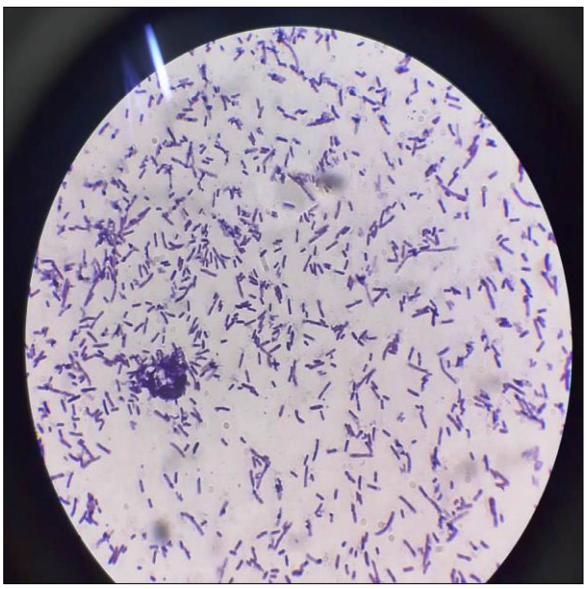

Figure 5 Microscopy of isolates: Gram positive bacilli with endospores under oil immersion (x100)

4. Discussion

In present study attempt was made to determine the bacterial load in the hospital sewage by MPN method, Serial tube dilution method and isolation of coliform bacteria by conventional bacterial identification methods.

Common physico-chemical parameters which affect the bacterial flora of the hospital sewage were determined. Temperature of sewage was recorded to be 28(±1.22)°c. The other parameters were found to be pH 6.52 (±0.10), TDS 251 (±1.41) mg/l, COD 96.9 (±0.82) mg/l and BOD 69.5 (±0.58) mg/l.

Hospital sewage comprises of variable physico-chemical characteristics with high BOD and COD values. [10]. Temperature varies with the geographical location, climate and time duration of the sample collection. A study in India shows pH of about 6.42, BOD ranging from 92.8 mg/l to 270 mg/l [11] and COD 1142 mg/l[12]. Some studies on hospital sewage from Asia show the TDS of about 119 mg/l. [13]. The pH value is consistent with the other referred studies while TDS is much higher, COD and BOD values far less as compared to the studies referred [11,12,13]

The Presumptive coliform isolation by MPN method indicated all 15 tubes positive (5-5-5) which represented MPN value of >1800 (>1.8x10^3) coliform per 100 ml as per the standard MPN table. Confirmatory MPN method showed results 5-1-0 which corresponds to 33 (3.3x10^1) coliform per 100 ml as per the standard MPN table.

A similar study on the sewage samples from six hospitals in India showed the concentration of total coliform ranging from 0.92x10^3 MPN/100 ml to 2.4x10^3 MPN/100 ml and fecal coliform ranged to 1.8x10^1 to 3.2x10^2. Our findings are consistent with the referred study.[11]

The serial plate dilution method showed bacterial count of 6x10^6 CFU/ml on Blood agar and 2x10^6 CFU/ml on MacConkey's agar. A study reported total heterotrophic bacterial counts ranging from 1.9 x 10^7 to 8.3 x 10^{12} CFU /ml. The result of bacterial count is lower as compared to the referred study. [11]

Growth on Eosin Methylene blue agar showed colonies with metallic sheen confirmed the presence of Coliform bacilli in the hospital sewage sample.

Bacterial isolation by studying the gram nature, colony characteristics and biochemical characteristics showed 5 isolates of which 3 were Gram negative bacilli, one Gram negative cocco bacillus and one Gram positive bacilli with spore. The five Isolates were identified as *Escherichia coli, Klebsiella pneumonae, Chromobacter violaceum, Pseudomonas aeruginosa and Bacillus subtilis* as per the biochemical characteristics. The Gram negative coliform like *Escherichia coli, Pseudomonas aeruginosa, Klebsiella spp*. And *Bacillus spp* have been reported to be commonly isolated in many of the studies conducted on bacterial isolates from Hospital waste water.[14,15,16] *Chromobacter violaceum* is a saphrophytic environmental bacterium found in soil and water. It is commonly isolated from municipal waste but is rarely pathogenic. *Escherichia coli, Klebsiella pneumonae, Pseudomonas aeruginosa and* Bacillus *subtilis* are common pathogenic bacteria associated with hospital environment indicating fecal contamination of water.[16] Their isolation and significantly higher counts in the hospital sewage indicate heavy pollution of hospital sewage. This can cause severe health problems as the heavily polluted hospital sewage from the District hospital Ratnagiri is discharged untreated directly in to the public sewer system posing threat to the local community coming in contact with the sewage and risk of polluting natural water bodies which are used for fishing, drinking and general purpose.

5. Conclusion

From the results and analysis of the study it can be concluded that the hospital sewage is significantly contaminated with the pathogenic coliform bacilli. It is a health threat as the untreated sewage is directly discharged in the public sewer which is unscientific practice. There is need of sewage treatment facility at the District hospital Ratnagiri which is populated with variety of patients. The regulations for handling and management of hospital wastes are needed to be followed strictly.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have no conflict of interest to declare.

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