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Research Article
Isolation and Characterization of Pathogenic Bacteria from Kundu River Water of Nandyal, Kurnool, Andhra Pradesh, India

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Abstract

Background and Objective: Kundu river water was used by local population for various domestic purposes previously. Now the water is getting contaminated with municipal sewage and industrial waste. Frequently, during rainy season or floods, Kundu river gets overwhelming water which cause severe damage to the local population through various infections. The main objective of the present study is to identify the pathogenic strains present in the kundu river water and how it will affect the human population using this water for various domestic purposes. **Materials and Methods:** The present study was performed by collecting the water samples from the Kundu river. The contaminating bacteria were isolated by following microbiological methods. The morphological and biochemical analysis was done by following standard procedures. Morphologically it has been observed in microscope and Gram staining was done to differentiate between Gram positive and negative. Biochemical analysis was done to identify the genus of the bacteria. Also done the antibiotic susceptibility test to know how harmful they can be if they infect the surrounding population. **Results:** Based on the morphological and biochemical analysis, it was found that the Kundu river water is highly contaminated with pathogenic bacteria. Most of the bacteria that were isolated and identified in this study belong to *Escherichia coli*, *Klebsiealla pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Shigella dysenteriae*. Many of these bacteria were showing resistance towards the antibiotics in the antibiotic susceptibility test. **Conclusion:** In the present study, it was identified that Kundu river water is contaminated with bacteria which may affect the population if they use this water for domestic purposes without proper treatment. Therefore, this type of research can be useful in bringing the awareness both in the Government and public regarding the safety precautions and measures to be taken to prevent contamination of water resources.

Key words: Biochemical analysis, antibiotic susceptibility, pathogenic bacteria, water treatment, gram staining

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

India has been gifted with many rivers and Himalaya mountains to meet majority of water requirements of the country. Water resources mainly accomplish the deeds such as drinking, domestic along with agriculture, aquaculture and in power generation. Therefore, water plays an important role in the lives of human beings. Out of many water resources, the resource that is used for drinking and domestic purpose should be taken into consideration seriously because of contamination problem. Now a days, the water is getting contaminated with several pathogenic microorganisms.

Water, as a solvent plays an important role in the metabolism of human beings. It is essential for digestion, absorption of food, transportation of nutrients and excretion of waste products. Hence, physico-chemical and microbiological quality of drinking water has become an indispensable tool to check before its use[1]. Water acts as a medium for various microorganisms and therefore plays a key role in spreading transferable diseases. The pathogenic bacteria that usually appear in water contamination include *Bacillus* sp., *Clostridium botulinum*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella* sp., *Salmonella* sp., *Staphylococcus aureus* and *Vibrio cholera*. Recent reports have shown the presence of pathogenic bacteria in drinking water which are responsible to water borne diseases[2,3]. Several review papers have also explained the presence of pathogenic bacteria in various water resources[4,5]. Contaminating pathogenic bacteria will cause several diseases such as diarrhoea and gastric disorders. Mostly the children get affected with these pathogens. As per WHO, nearly 3.4 million people die every year due to the diseases related to water contamination[6]. As per UNICEF, approximately 4000 children die every day due to water contamination[7]. Water contamination not only affects the developing countries but is also a major problem in developed countries. Thus, contamination of water resources with pathogenic bacteria leading to illness is a major concern throughout the world. Improving water quality is the major parameter to reduce the water related diseases.

Kundu river is a branch of Penna river in the Rayalaseema region which serves as a water resource for agriculture, frequently gets overflowed in the rainy season due to floods and brings heavy loses to the local community. Now it has become a destiny to discharge drainage water and industrial waste without prior treatment which has become a house to pathogenic bacteria. Hence there is a need to know the contaminating organisms and the level of contamination responsible for causing illness in the surrounding population. The present study is aimed to identify the bacteria prevailing in Kundu river.

MATERIALS AND METHODS

Study area: Kundu river is located in Rayalaseema region of Andhra Pradesh, India. Its geographical coordinates are 15°29'0" North, 75°29'0" East. The temperature is about 28-43°C and the population is about 2,00,516.

Collection of sample: In the month of January, 2017, the water sample was collected aseptically in a sterile bottle with cap. The bottle was labelled with full details and stored in clean place.

Culture media conditions: Nutrient broth media was prepared by adding 0.5% peptone, 0.3% yeast extract, 0.5% NaCl, 0.25% glucose and pH was adjusted to 7 at room temperature. When the nutrient agar plates were required, approximately 1.5% agar was added to nutrient broth.

Isolation of bacteria from Kundu river water sample: From the collected water sample, 1 mL was taken and it is serially diluted with distilled water. The serial dilution was done up to 10^{-5}. From each dilution, 100 µL of sample was spread on nutrient agar plate. The plates were incubated at 37°C for 24 h. The colonies appeared on nutrient agar plates were sub-cultured. The sub-cultures were maintained on nutrient agar plates for routine use and stored at 4°C. The main cultures were preserved in 20% glycerol vials at -20°C.

Characterization of bacterial culture

Morphological studies

Gram staining of bacteria: To differentiate the bacteria between Gram-positive or Gram-negative, grams staining was done. Made a thin smear of culture on glass slides, dried the smear and heat fix, covered the smear one by one with crystal violet (60 sec), gram's iodine (60 sec), 95% ethanol (20 sec) and safranin (40 sec). Air dried the slides after washing with distilled water and observed under microscope (Olympus, MLX-B Plus, India).

Shape, size, colour: Morphological characteristics such as shape, size and colour were studied by microscopic observation. The shape of the colony was studied by observing their margin and elevation. The size of the bacteria was studied by microscopic observation and it can be calculated in microns. The size of the colony was measured by scale. The colour of the bacteria was determined by observing the colony under microscope.

Endospore staining: Endospores staining was done by preparing a thin smear of bacteria on a glass slide and then

flooded with 5% malachite green. The glass slides were then heated to steaming for about 5 min. Glass slides were washed with distilled water and counter stained with 0.5% safranin for 30 sec. Finally, the slides were washed with distilled water and observed under microscope.

Biochemical tests: Biochemical analysis was done in order to know which types of bacteria are present in the current water samples of the study. The biochemical tests include amylase test, catalase test, citrate test, gelatine test, indole test, methyl red test, nitrate reduction test, oxidase test, sugar fermentation test, urease test and voges proskauer test. These tests were performed according to standard methods.

Antibiotic sensitivity assay: Antibiotic sensitivity was done by cup assay method. Overnight culture of the test organism was spread on nutrient agar plates and the plates were incubated at 37°C for 16 h. By using sterile borer, on each plate separate cups were made for pouring antibiotics separately. Each antibiotic at their respective concentrations (ampicillin (10 µg), azithromycin (15 µg), chloramphenicol (10 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamycin (20 µg), neomycin (30 µg), streptomycin (25 µg), tetracycline (30 µg) and vancomycin (30 µg)) were poured on cups of pre-inoculated plates. All the plates were incubated at 37°C for 48 h. The inhibition zone around each cup was observed against each antibiotic and also against each bacterium. Plates without any antibiotics concentration were treated as reference plates.

RESULTS

The aim of the study was to isolate and identify the bacteria exist in the water samples collected from Kundu river. Further the isolated bacteria were tested for antibiotic susceptibility.

Serial dilution and spread plate method: Serial dilution method was followed in order to reduce the number of colonies and also to isolate the colonies purely. In the present study, a total of six colonies were isolated and they were maintained on nutrient agar plates, stored at 4°C for regular use. All six isolates were designated as KRW 1, KRW 2, KRW 3, KRW 4, KRW 5 and KRW 6 (Fig.1, 2). The KRW is abbreviated as Kundu river water. The pure cultures of all six isolates were preserved in glycerol stocks at -20°C for further use.

Morphological characterization of bacteria isolated from Kundu river water sample: In the present study, the morphological characteristics such as colour, size, shape and nature of colonies of all 6 isolates were determined by growing them on nutrient agar plates. In addition, all the isolates were tested by gram's staining and endospore staining to check whether they belong to Gram-positive or Gram-negative. The details of the morphological characteristics of all 6 isolates were shown in Table 1. Most of the colonies isolated during this study were irregular in nature, size ranges from 2-4 µm, colour was generally white to cream and their shape is circular to rod. Out of six isolates isolated from Kundu river water four strains were Gram-negative rods, one is Gram-positive rod and another one is Gram-positive cocci (Fig. 3). The bacteria isolate KRW1 appeared as white Gram-positive rod shaped motile bacteria. KRW 2, 3 and 4 though appeared different in terms of colour but all three are Gram-negative rod shaped bacteria. KRW 5 appeared as light brown in colour and is Gram-positive cocci. KRW 6 which appeared as cream in colour is a Gram-negative rod. Morphologically they appear as either Gram-positive rod shaped or cocci and Gram-negative rods.

Biochemical characterization of bacteria isolated from Kundu river water sample: In the present study, 6 colonies were isolated from Kundu river water samples. The isolates were designated as KRW 1-6. All these isolates were characterized biochemically. Biochemical characterization of KRW 1-6 samples showed discrepancy in their response to different biochemical tests. KRW 1 isolate showed positive results to all biochemical tests except indole, methyl red and urease test. KRW 2 isolate showed negative result to citrate, gelatin, oxidase, urease and voges proskauer test. Similarly, KRW 3 showed negative result to gelatin, indole, methyl red

Table 1: Morphological characteristics of the isolates

	Colony characteristics			Cell features		
Isolate No.	Colour	Size (µm)	Nature of colony	Gram's staining	Endospore staining	Test organisms identification
KRW 1	White	4	Dry, Flat, Irregular	+ve, rod	+ve	*B. subtilis*
KRW 2	Cream	3	Shiny, Mucoid, Entire	-ve, rod	-ve	*E. coli*
KRW 3	White	2	Transparent, Mucoid, Flat	-ve, rod	-ve	*K. pneumoniae*
KRW 4	Light blue	4	Smooth, Flat, Filamentous, Mucoid	-ve, rod	-ve	*P. aeruginosa*
KRW 5	Light brown	4	Smooth, Flat, Convex, Circular	+ve, Cocci	-ve	*S. aureus*
KRW 6	Cream	2	Smooth, Rod, Entire	-ve, rod	-ve	*S. dysenteriae*

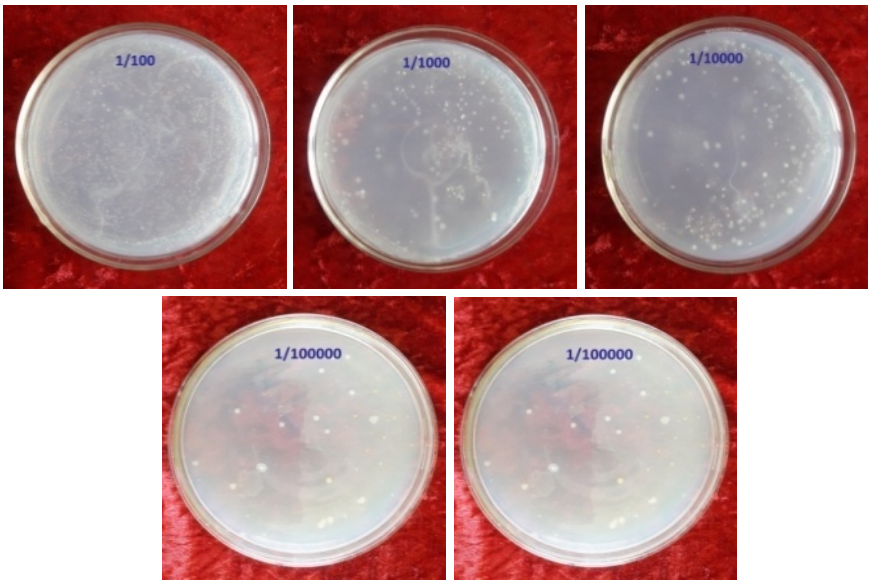

Fig. 1: Spreading of serially diluted Kundu river water samples showed mixed cultures

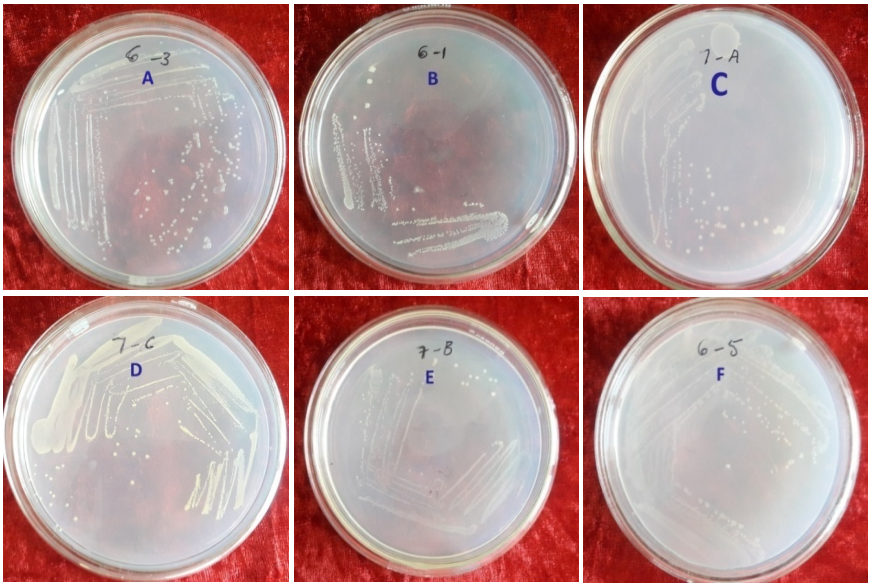

Fig. 2: Maintenance of isolates of Kundu river water sample (KRW) on nutrient agar plates
A: KRW 1, B: KRW 2, C: KRW 3, D: KRW 4, E: KRW 5, F: KRW 6

and oxidase tests. KRW 4 showed negative result to amylase, indole, methyl red, sugar fermentation, urease and voges proskauer test. KRW 5 isolate showed positive for all tests except indole and oxidase test whereas KRW 6 showed positive result to amylase, gelatin, methyl red and sugar fermentation tests and remaining all are negative. The biochemical characteristics of all the 6 isolates were done by performing biochemical tests as mentioned in materials and methods. The results of the biochemical tests were shown in Table 2.

Antibiotic sensitivity test: All 6 isolates have been checked for antibiotic sensitivity. A set of antibiotics available in the market have been used in the present study. Each isolate has been checked with all antibiotics. Almost all isolates have shown sensitivity to some antibiotics and resistance to

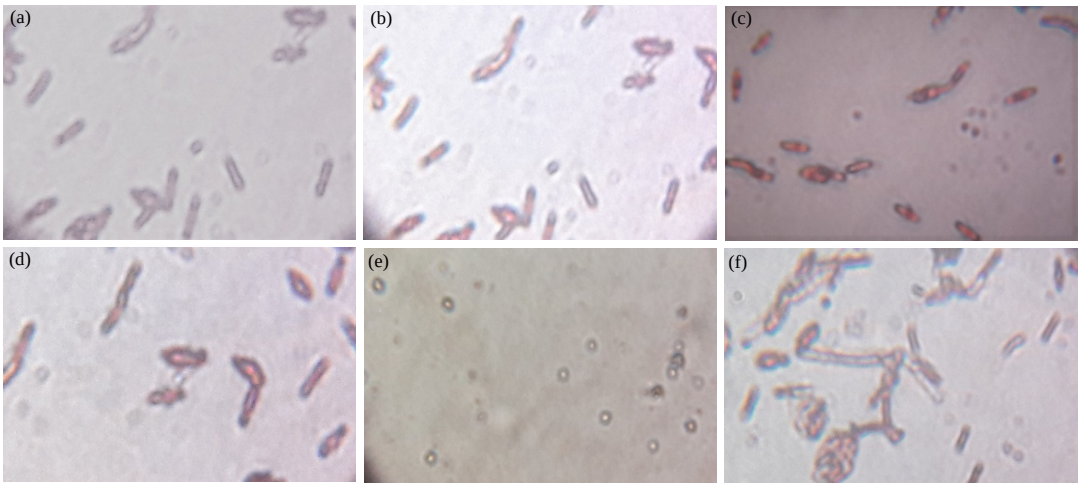

Fig. 3(a-f): Gram staining, (a) KRW 1: G+ve, rods, (b) KRW 2: G-ve, rods, (c) KRW 3: G-ve, rods, (d) RW 4: G-ve, rods, (e) KRW 5: G+ve, Cocci and (f) KRW 6: G-ve, rods

Table 2: Biochemical characteristics of the isolates

Name of the test	KRW 1	KRW 2	KRW 3	KRW 4	KRW 5	KRW 6
Amylase test	+	+	+	-	+	+
Catalase test	+	+	+	+	+	-
Citrate test	+	-	+	+	+	-
Gelatin test	+	-	-	+	+	+
Indole test	-	+	-	-	-	-
Methyl red test	-	+	-	-	+	+
Nitrate reduction test	+	+	+	+	+	
Oxidase test	+	-	-	+	-	-
Sugar fermentation test	+	+	+	-	+	+
Urease test	-	-	+	-	+	-
Voges Proskauer test	+	-	+	-	+	-

+: Positive, -: Negative

Table 3: Antibiotic sensitivity tests for identified microorganisms

Antibiotics	KRW 1	KRW 2	KRW 3	KRW 4	KRW 5	KRW 6
Ampicillin (10 µg/disc)	S	S	S	R	S	R
Azithromycin (15µg/disc)	R	R	R	R	R	R
Chloramphenicol (10 µg/disc)	R	R	R	R	R	R
Ciprofloxacin (5µg/disc)	S	S	S	S	R	S
Erythromycin (15µg/disc)	S	S	S	S	R	S
Gentamycin (120 µg/disc)	S	S	S	S	S	S
Neomycin (30 µg/disc)	R	R	R	R	R	R
Streptomycin (25µg/disc)	R	R	R	R	R	S
Tetracycline (30 µg/disc)	S	S	S	S	S	S
Vancomycin (30 µg/disc)	S	S	S	S	S	S

S: Sensitive, R: Resistant

some other antibiotics but the isolate KRW 5 has shown resistance to many antibiotics compared to other isolates. The response of the isolates to different antibiotics were shown in Table 3. In the present study, no statistical analysis was done for antibiotic sensitivity, only tested whether they are sensitive or resistant. If the zone around the antibiotic cup appears it was taken as sensitive and it if zone, then it is resistant.

DISCUSSION

In the present study, it was identified that the Kundu river water is contaminated with several bacteria. Based on the morphological and biochemical characteristics the bacteria isolated from Kundu river water, KRW 1-6 were tentatively identified as *Bacillus subtilis*, *Escherichia coli*, *Klebsiealla pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus*

aureus and *Shigella dysenteriae*, respectively by referring to Bergey's manual of determinative bacteriology. All these bacteria isolated in the present study belong to the genus of pathogenic bacteria.

Most of these pathogenic bacteria are responsible for water-borne diseases such as gastrointestinal disorders and diarrhoea. The ingestion of pathogenic bacteria through water contamination may lead to epidemics in that particular locality or geographical region[8,9]. Every year millions of people die in developing countries mainly due to the diseases caused by water contamination[10]. Water contamination with pathogenic bacteria is a serious concern not only in developing countries but also in developed countries[11]. Most of the bacteria cause gastrointestinal disturbances having symptoms such as diarrhoea, fever, vomiting and abdominal pain. Most of the above symptoms were caused by *Shigella* spp. and *E. coli*. The *E. coli* isolates observed in the present study were predominant pathogens in urinary tract infections, gastroenteritis and neonatal meningitis[12,13] whereas *K. pneumonia* cause necrosis, inflammation and haemorrhage and *S. dysenteriae* cause dysentery, haemorrhagic colitis, pyogenic infections, septicaemia and also urinary tract infection.

Many of the water resources not only in developing countries but also in developed countries is prone to contamination with different types of pathogenic microorganisms. Mainly these organisms include *E. coli*, *Shigella*, *Salmonella* and some other depending up on the microorganisms prevailing in that locality. Many of these bacteria have gained resistance against several antibiotics available in the market. It is better to reduce the use of antibiotics frequently otherwise as these pathogenic microorganisms slowly develop resistance and thereby they are not eliminated easily[14-18].

The present study highlights the presence of pathogenic bacteria in Kundu river water. Therefore, care should be taken to flee from the infections caused by these pathogenic bacteria especially at the time of rainy season or during floods as this river overflows and enter the living environment. It is better to have a water treatment process before they were used for domestic purposes. The limitation of the present study is the bacteria isolated were not identified phylogenetically.

CONCLUSION

This study clearly recommended that the river area is highly contaminated by pathogenic bacteria that too mainly with Gram-negative rods. If this water is used for domestic purposes without any proper treatment it may leads to severe illness. This will affect mainly children living in that particular geographical area. It recommends the need for establishing a proper treatment plant to remove all contaminated bacteria before they have been utilized for domestic purposes.

SIGNIFICANCE STATEMENTS

This study discovers the presence of pathogenic bacteria in the river water which is contaminated with municipal and industrial wastes. The contaminating pathogenic bacteria include the strains from the genus of *Escherichia*, *Pseudomonas*, *Staphylococcus* and *Shigella*. This study will provide information to the researchers about the existence of the pathogenic bacteria in Kundu river. It also brings awareness in the society about the level of contamination and how harmful it is to human beings living around that environment. Thus, the new theory on existence of pathogenic bacteria in Kundu river demands the emergency action to be taken towards preventive measures.

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