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To study the prevalence and drug resistance in *Pseudomonas aeruginosa* from drinkingwater sources of Morbe Dam, Navi Mumbai

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Abstract:
A pathogen's multiple drug resistance (MDR) is becoming a common issue and it is seen in many species, which makes organisms immune to antibiotics. MDR affects not only hospitals but they are becoming common in environmental stress also, so it can easily affect humans and animals which is very dangerous for living welfare. While chlorination reduces microorganisms in drinking water, they can survive the process of treatment and reach the delivery system. In addition, the antibiotic resistance has been recorded before in microorganisms. [1] [2] [3]. The risk associated with microbe pathogenicity is increased through its ability to resist antibiotic treatment. In waste water treatment facilities, the use of biological procedures may cause a selective rise in bacteria resistant to antibiotics, increasing the prevalence of multi-drug-resistant species.[4] Considering the very fact that the general community's public health could also be associated with the standard of treated waste water supplied which public health are often at order of protecting drinking water from contaminants, the current project aims to separate Pseudomonas bacteria from the surface water at Morbe Dam. Mumbai Navi. Another objective was the use of their antibiotic resistance profiles to characterise the isolates.

Keywords: MDR (Multiple Drug Resistance), Prevalence, physiochemical analysis, Heavy metal analysis, Isolation, Antibiotic susceptibility test, 16s RNA gene, PCR (polymerase chain reaction).

Introduction:

It is known that water serves as a medium for the growth and spread of related human microbes [5]. It has long been acknowledged that water is essential for preventing disease (Hofkes, 1981; WHO, 1996). Water is a development factor in almost every industry, including manufacturing, services, and agriculture (UNESCO, 2006).

The claim that "safe drinking water is the birth right of all human beings, as much a birth right as clean air" emphasises the importance of this. Additionally, it stated that up to 6 million children perish every day from waterborne illnesses connected to a lack of access to clean drinking water or proper sanitation, and that the majority of people on the planet, particularly in most of Africa and Asia, lack access to safe drinking water.[5] [6] [7]

(WHO 2004) stated that diseases caused by drinking water contamination are a serious burden on human health, and that initiatives to improve drinking water quality offer significant health benefits. Most communities rely on pipe-borne water from municipal water treatment plants as their primary supply of clean drinking water. Many water treatment plantseither do not supply or fail to fulfil the water needs of the community they serve owing to corruption, a lack of maintenance, or an increase in population. Because of the lack of piped water, communities have turned to groundwater sources, which are readily available. Wells are a typical source of ground water that can be used to supplement communal water suppliesor make up for shortfalls.[6]

Disease and Prevalence

Pseudomonas aeruginosa is a prevalent cause of UTIs, as well as skin and soft tissue infections. [8] In India, *p. aeruginosa* infection prevalence rates range from 10.5 percent to 30 percent. In a multicentric study performed by Ling J M et al, it ranged from 3 to 16 percent. [8]

Methodology:

Site observation details: Important observations made around the sampling sties. The field records for the following environmental parameters were recorded.
(visual clarity, weather condition, presence of animals, and other comments.)

Sample collection of water from morbe dam: Water samples were collected from

Morbe dam, Navi Mumbai, in triplicate. Water samples were obtained at a depth of one metre. from the surface site per sampling in suitable sterile container from the selected hotspots during the below.

SrNo.	Months ofsample collection	Quantity (ml)	Sampling Frequency
1	March 2020	50 ml	MGMCRLDW07 MGMCRLDW07(3 March 2020) MGMCRLDW07(10 March 2020) MGMCRLDW07 (18 March 2020)
2	July 2020	50 ml	MGMCRLDW08 MGMCRLDW08 (3 July 2020) MGMCRLDW08 (15 July 2020) MGMCRLDW08 (25 March 2020)
3	December 2020	50 ml	MGMCRLDW09 MGMCRLDW09 (10 December 2020) MGMCRLDW09 (19 December 2020) MGMCRLDW09 (29 December 2020)

Table 1: Sampling frequency

Where,
MGMCRLDW (07), (08), (09) =
MGM = Name of the Institute
CRL = Name the laboratory (central research laboratory)
DW= Dam water
07, 08, 09: code of water sample collected
A, B, C: triplicate of water sample collected

Physiological Characteristics water:

Collected water sample from Morbe Dam was checked against following physiochemical characteristics to determine the physical characteristics of collected water are time, temperature, pH, colour, cell density.

Heavy metal analysis:

A heavy metal is defined as any metallic chemical element with a high density that is hazardous or poisonous at low doses. Heavy metals include lead (Pb), cadmium (Cd), arsenic (As), chromium (Cr), thallium (Tl), and mercury (Hg).[10]

Isolation and Characterization of Isolates:

All Gram-negative organisms were identified using standard methods such as Gramme staining, indole negative, catalyse positive, oxidase positive, and gramme nature negative tests, among others, while the gram-negative organism was identified using the himedia kit.

Antibiotic susceptibility test:

The disk-diffusion test, commonly known as the Kirby-Bauer test, is the most popular antibiotic susceptibility test for determining which antibiotic should be used to treat an infection. This approach is focused on measuring bacterial growth inhibition under standard conditions. Taken a Hardy Diagnostic Saline 0.85 percent, 1.8mL Selected multiple Colonies were separated from the agar surface using a 1ul loop.

If the turbidity is equal, proceed with the Mueller Hinton Plate inoculation. If not, adjust the turbidity by introducing more isolated colonies in the same way if the turbidity is lower than normal, or add more saline if the turbidity is higher. When the turbidity is similar to the norm, inoculate the designated Mueller Hinton plate. The pseudomonas aeruginosa isolates were tested against twelve antibiotics, which are as follows:
Amoxiclav(30mcg),

Tobramycin(10mcg), Cefazolin(30mcg), Gentamicin(10mcg), cefuroxime(30mcg),
Amoxiline(30mcg), Cefotaxime(30mcg), Ciprofloxacin(5mcg), Tetracycline(30mcg),

Cefoperazone(30mcg), Ceftazidime(30mcg), Ofloxacin(5mcg). Antibiotic discs were put aseptically using sterile forceps and plates were incubated at 37°C for 24 hours [24].

Polymerase chain reaction (PCR)by using 16s RNA sequencing:

Bacterial species recognition is widely used in microbial ecology to evaluate the biodiversity of environmental samples, as well as in medical microbiology to diagnose infected patients. The 16S rRNA gene is sequenced using polymerase chain reaction (PCR), and the DNA is sequenced thereafter. PCR is a molecular biology technique that is used to amplify individual DNA fragments using a sequence of cycles that include:

1. Double-stranded DNA template denaturation
2. Annealing of complementary primers (short oligonucleotides) to the template
3. Primer extension by the DNA polymerase enzyme, which produces a new DNA strand

Result and Discussion:
Site observation details:

Fig. 1: Some selected locations from Morbe Dam in the month of (a)March 2020 (b) July 2020 (c)December

	March 2020		
Sample	MGMCRLDW07 (3 March 2020)	MGMCRLDW07 (10 March 2020)	MGMCRLDW07 (18 March 2020)
Turbidity	Clear	Clear	Clear
Debris	No	No	No
Weather condition	Sunny (30.7 ± 2°C)	Sunny (30.7 ± 2°C)	Sunny (30.7 ± 2°C)
Wind flow	Yes	Yes	Yes
Rain fall	No	No	No
Presence of animal	Yes	No	Yes
Other parameters	Cemetery Faecal contamination	Cemetery	Cemetery Faecal contamination Waste dumping

Table 2: Site observation detail in month of March 2020

The collected water sample in month of March 2020 from Morbe dam, it was clear with no turbidity and debris, there was presence of faecal contamination, animals and cemetery as shown in table 2.

	July 2020		
Sample	MGMCRLDW08 (3 July 2020)	MGMCRLDW08 (15 July 2020)	MGMCRLDW08 (25 July 2020)
Turbidity	Turbid	Turbid	Turbid
Debris	Yes	Yes	Yes
Weather condition	Cloudy (26.2 ± 2°C)	Cloudy (26.2 ± 2°C)	Cloudy (26.2 ± 2°C)
Wind flow	Yes	Yes	Yes
Rain fall	Yes	Yes	Yes
Presence of animal	No	No	No
Other parameters	Cemetery	Cemetery	Cemetery Faecal contamination

Table 3: Site observation details in month of July 2020

The collected water sample in month of July 2020 from Morbe dam, it was turbid with presence of debris, there was presence of faecal contamination, animals and cemetery as shown in table 3.

	December 2020		
Sample	MGMCRLDW09 (10 December 2020)	MGMCRLDW09 (19 December 2020)	MGMCRLDW09 (29 December 2020)
Turbidity	No	No	No
Debris	Yes	Yes	Yes
Weather condition	Cloudy (22.9 ± 2℃)	Cloudy (22.9 ± 2℃)	Cloudy (22.9 ± 2℃)
Wind flow	Yes	Yes	Yes
Rain fall	No	No	No
esence ofanimal	Yes	Yes	Yes
Other parameters	Cemetery Faecal contamination	Cemetery Faecal contamination	Cemetery Faecal contamination

Table 4: Site observation details in month of December 2020

The collected water sample in month of December 2020 from Morbe dam, it was clear with presence of debris, there was presence of faecal contamination, and cemetery as shown in table 4.

Collection of water sample:

Fig.2: Water Sample collection from Morbe dam

Sample were collected from Morbe dam, water samples were collected at a depth of 1m fromthe surface per site per sampling in suitable sterile container from the selected different 3 hotspots in 3 different seasons summer, spring, winter.

Sr no	Sample no	During months
1	MGMCRLDW07	10 March 2020
2	MGMCRLDW08	18 July 2020
3	MGMCRLDW09	29 December 2020

Table 5: Sample collection of water

Physiological characteristics of water sample:

Water sample was collected from Morbe Dam, in three different seasons: March 2020, July 2020, December 2020. Collected sample was then checked for various parameter such as temperature, pH, colour, turbidity, as shown in table no 2, 3, 4.

pH of water sample:

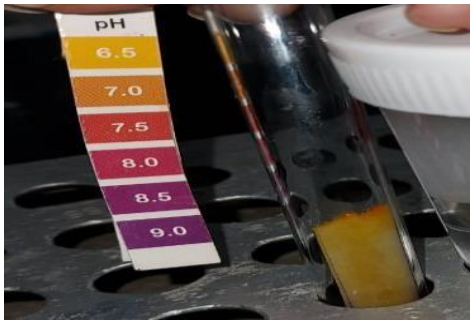


Fig. 3: Representative figure of pH of water sample

Sr. no	Parameters	Physiological analysis (March 2020)		
1.	Sample code	MGMCRLDW07 (3 March 2020)	MGMCRLDW07 (10 March 2020)	MGMCRLDW07 (18 March2020)
2.	Water collection time	3:30 PM	3 PM	4 PM
3.	Temperature	(30.7 ± 2 º C)	(30.7 ± 2 º C)	(30.7 ± 2 º C)
4.	pH	7.2	7.2	7.2
5.	Color	Colorless	Colorless	Colorless
6.	Turbidity	Less turbidity	Less turbidity	Less turbidity

Table 6: Result for physicochemical analysis for March 2020

Water samples were obtained from three separate locations in March 2020, between 3 and 4 p.m. The collected water had a temperature of 30ºC, a pH of 7.2, was colourless, and was lessturbid.

Sr. No	Parameters	Physiological analysis (July 2020)		
1.	Sample code	MGMCRLDW08(3 July2020)	MGMCRLDW08(15 July 2020)	MGMCRLDW08 (25 December 2020)
2.	Water collection time	3:30 PM	3 PM	4 PM
3.	Temperature	(22.9 ± 2 º C)	(22.9 ± 2 º C)	(22.9 ± 2 º C)
4.	pH	7.2	7.0	7.2
5.	Color	Muddy	Muddy	Muddy
6.	Turbidity	Turbidity	Turbidity	Turbidity

Table 7: Result for physicochemical analysis for July 2020

Water samples were obtained from three separate locations in July 2020, between 3 and 4 p.m. The collected water had a temperature of roughly 30ºC, a pH of around 7.2, and wascolourless and turbid.

Sr.no	Parameters	Physiological analysis (December 2020)		
1.	Sample code	MGMCRLDW09 (10 March 2020)	MGMCRLDW09 (19 December 2020)	MGMCRLDW09 (29 December 2020)
2.	Water collectiontime	3:30 PM	3 PM	4 PM
3.	Temperature	(26.92 ± 2 º C)	(26.92 ± 2 º C)	(26.92 ± 2 º C)
4.	pH	7.2	7.0	7
5.	Color	Clear	Clear	Clear
6.	Turbidity	Less Turbidity	Less Turbidity	Less Turbidity

Table 8: Result for physicochemical analysis for December 2020

Water samples were obtained from three distinct places in December 2020, about 3-4 PM.The collected water had a temperature of 30ºC, a pH of 7.2, was colourless, and was less turbid.

Heavy metal analysis

The ICPOES instrument was used for to determine the presence and amount heavy metals in Morbe dam water. Mercury (Hg), Mercury (Hg), Cadmium (Cd), Arsenic (As) these heavy metals were found in water samples were <0.001 ppm, <0.001 ppm, <0.001 ppm, <0.001ppm were respectively. all the tested sample showed no heavy metal were given below in table 9. The obtained results of trace metal analysis were in similar lines of Hasan et al, 2016. [19] These levels are acceptable and water safe consumption.

Sr. no	Parameters	Result
1	Mercury (Hg)	< 0.1 ppm
2	Lead (Pb)	< 0.1 ppm
3	Cadmium (Cd)	< 0.1 ppm
4	Arsenic (As)	< 0.1 ppm

Table 9: Representative result of all 3 samples of Heavy metal analysis

Isolation and characterization of bacteria:

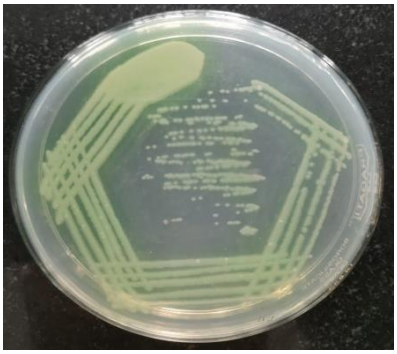


Fig. 4: Bacterial Colonies in Cetrimide Agar

The collected water sample in 3 different seasons at different time point, of the dam were streaked on the nutrient agar by streak plate method to isolate the targeted bacteria. The following representative figure for inoculated master plate.

Colony characteristic of MGMCRLDW07, MGMCRLDW08, MGMCRLDW09 on cetrimide agar plate.

Characteristic on nutrient agar plate	MGMCRLDW07 March 2020	MGMCRLDW08 July 2020	MGMCRLDW09 December 2020
Size	Small colonies	Small colonies	Small colonies
Shape	Round	Round	Round
Colour	Green	Green	Green
Consistency	Sticky	Sticky	Sticky
Transparency	Opaque	Opaque	Opaque
Margin	Entire	Entire	Entire
Elevation	Raised	Flat	raised
Gram nature	Gram negative rods	Gram negative rods	Gram negative rods

Table 10: Colony characteristic of isolate

Most strains of *pseudomonas aeruginosa* produce colonies bluish-green in colour, sticky colonies, with smooth surface and entire edge in cetrimide agar. [20] as result of inoculation of all morphologically similar colonies to *pseudomonas aeruginosa* were found in all 3 season samples which were MGMCRLDW07, MGMCRLDW08, MGMCRLDW09.

Gram staining:

Pseudomonas aeruginosa is gram-negative bacteria, so we sub cultured gram-negative bacilli bacterial colonies which were confirmed by gram staining in order to get pure culture were under 100 x oil immersion microscope. Representative figures of gram-negative bacilli bacteria were given below.

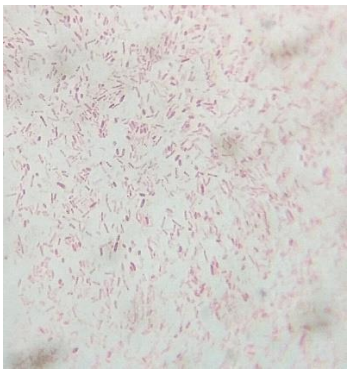
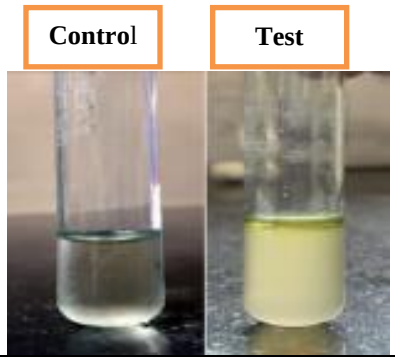
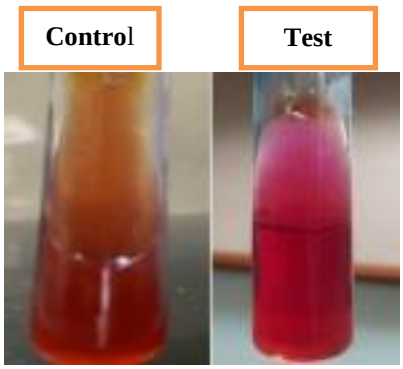
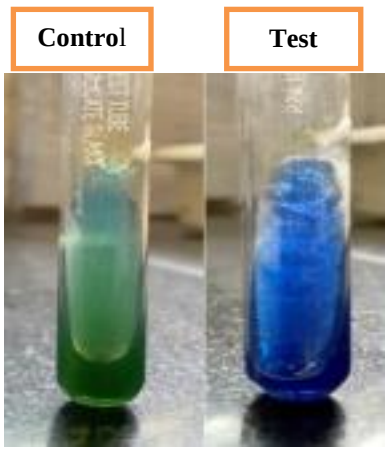
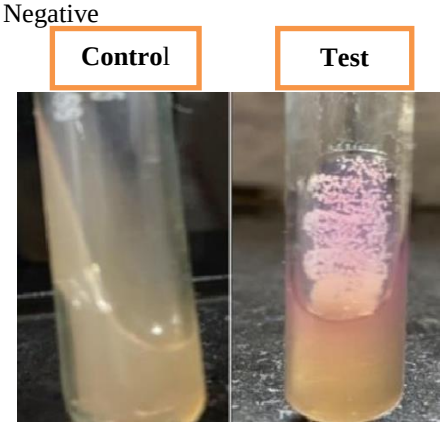
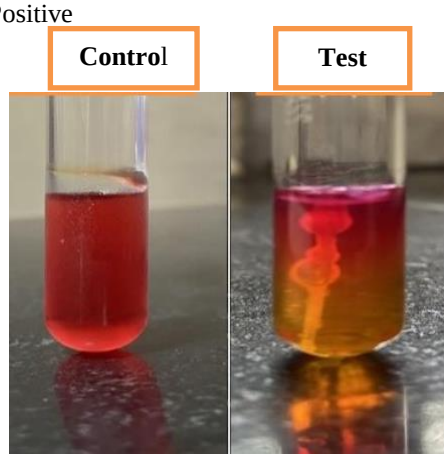
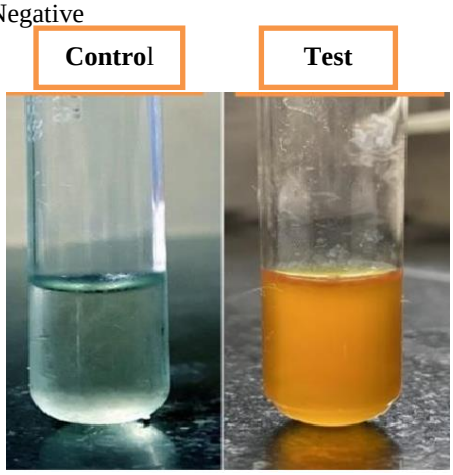
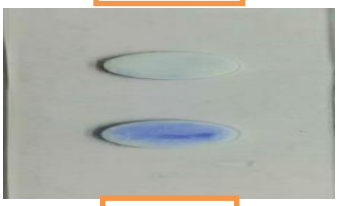


Fig. 5: Gram staining for isolates

The given observation showed its gram negative rod shaped bacteria. It possible it may be presence of *pseudomonas aeruginosa*.but the will confirm further process biochemical test.

Tests	Observations	Interpretation
Indole test	Negative 	MGMCRLDW09 does not produce theenzyme tryptophanase which determinates the amino acidtryptophan to give indole ring. This indole ring formed reacts with Kovacs reagent to produce red colour ring at the interphase. Since no red colour wasformed in the reagent layer at the top of the broth it can be concluded that the organism gives Indole negative test.
TSI test	k/k no gas no H2S produced 	MGMCRLDW09 could not utilize any carbohydrates i.e., glucose, sucrose, lactose, present in the medium, only peptone was utilized. The alkaline chemicals produced by the breakdown of peptone increased the pH of the medium, resulting in a colour change in the pH indicator phenol red from orange to pink.
Citrate test	Positive 	MGMCRLDW09 produces the enzyme C*itrase permease* which metabolizes the citric acid used in the Simmons's citrate slant. This utilization of citrate by the bacteria produces alkaline compounds in the medium leading to change in the pH of the medium from neutral to alkaline. This increase in pH causes the pH indicator, Bromothymol blue, to shift colour from green to blue.
Urease test	Negative 	The MGMCRLDW09 urease negative test reveals that bacteria hydrolyse urea, producing ammonia and CO2. The formation of ammonia alkalises the media, and the pH changes from light orange at pH 6.8 to magenta (pink) at pH 8.1 by altering the colour of phenol red. Urease-negative bacteria do not change colour or turn yellow when acid is produced.

Motility test	Positive 	MGMCRLDW09 is a motile organism since the bacteria showed diffused growth in the semi-solid media. The organism showed swarming motility. The mannitol present in the medium was utilized by the bacteria as the pH indicator (phenol red) added in the medium changed its colour from red toyellow.
Methyl red test	Negative 	MGMCRLDW09 gives negative MR- test which is an indicative that the organism does not utilize the glucose to perform mixed-acid fermentation. The broth turned orange-yellow on addition of the pH indicator Methyl red, as glucose was not fermented it can be concluded that the change in the colour of the broth was due to the utilization of peptone incorporated in the media which lead to alkalinity of the medium. The pH range of the Methyl red indicator is Red=below pH 4.4 and orange/yellow =pH 4.4-pH 6.2.
Catalase test	Positive 	MGMCRLDW09 gives catalase positive reaction as it showed rise of effervescence when the bacterium was inoculated in 3% H_2O solution. This indicates that the organism produces the enzyme catalase which helps it to scavenge to harmful oxygen derivatives also known as reactive oxygen species such as H2O2, which are formed by auto-oxidation of enzymes carried out by neighbouring aerobic cells.
Oxidase test	Positive 	MGMCRLDW09 generates an oxidase positive reaction; organisms with cytochrome c in their respiratory chain are oxidase positive, which turns the reagent blue/purple.

Isolate from MGMCRLDW07(A, B, C), MGMCRLDW08(A, B, C), MGMCRLDW09 (A,B, C) showed, Citrate utilization test, Mannitol test, and catalase test, oxidase test were positive. whereas indole test, urease test, methyl red test negative & k/k with no production ofgas was seen in TSI test.

By using, Bergey's Mannual, the preliminary identification of the isolates was done

and the isolate were identified up to genus level *pseudomonas aeruginosa*, and by using Bergey's manual it was concluded that isolated microorganism can be *pseudomonas aeruginosa*.

Molecular detection:

DNA extraction:

DNA extraction was done from pure isolated colonies from sample MGMCRLDW09 usinghimedia's HipurA bacterial genomic DNA kit.

DNA amplification was done by PCR and the results was analysed by agarose gel electrophoresis technique (AGE).

Polymerase Chain Reaction:

PCR was set with 16s primers and loaded on 2% agarose gel for QC.

Five microliters of the product were loaded along with molecular marker 100-1000bp.

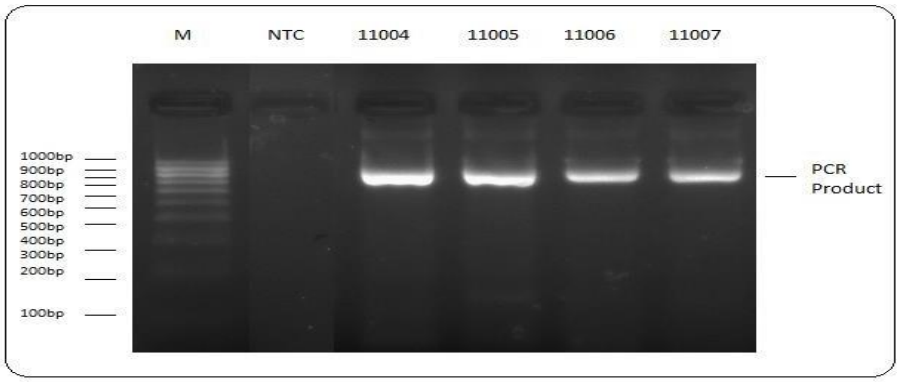


Fig. 6: PCR product separationSequence of the bacterial gene:

As the result of 16s rRNA sequencing which were
>11006(806R) is the MGMCRLDW09

ATATAAGGAGGTTTTGGTTCTGGCTTCAGAATTGAAACGCTGGGCGGGCAGGCTC
TAACACTTGCAAAGTCGAGCGGATGAAGGGGAAGCTTGCTCCGGGATTCAGCGG
GCGGGACGGGTGAAGTAAAGCCTAAGGAAATCTGCCTGGTAGTGGGGGATAACG
TCCGGAAACGGGCGCTAATACCGCCTACGTCCTGAGGGAGAAAGTGGGGGATCT
TCGGACCCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTA
AAAGGCCTACCAAGGCGACGATCCCGTAACTGGTCTGAGAGGATGATCAGTCAC
ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC
GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGT
TTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGT
AATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGG
TGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCA
AAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTG
AATTTTTTATATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGA
TACTGACACTGAGTTCCATAAATTTATTTAA

In order to identify the gene sequence of the isolated organism by using Nucleotide BLAST was performed, and the result shown below.

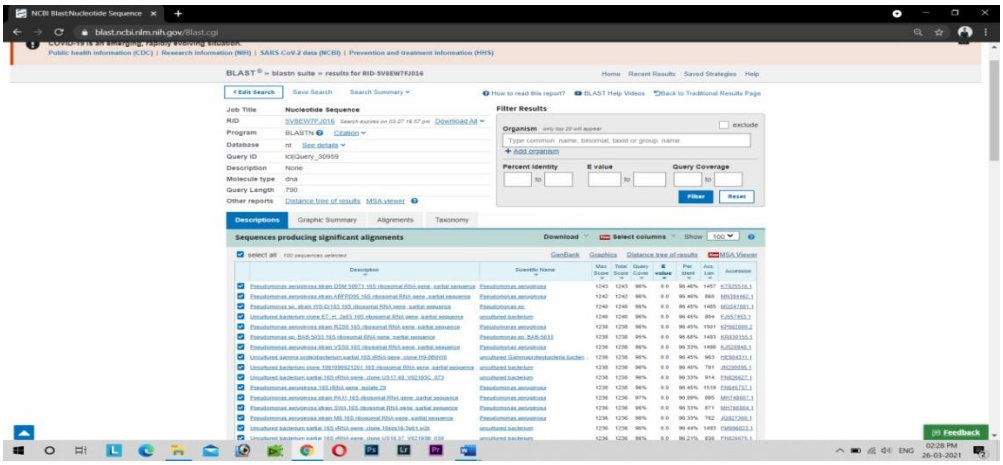


Fig. 7. Gene Sequence of the isolated organism by using blast

A bacterial strain called MGMCRLDW09A was discovered from a water sample obtained at Morbe Dam. Genomic DNA was taken from the isolated bacterial strain and amplified and sequenced using the primers. A total of 1.183 bp from the 16s rDNA gene were sequenced and utilised to identify the isolated bacterial strain. Comparing these test sequences to GenBank's redundant collection. [21] database was searched using BLAST [22][23]. The BLAST results revealed that the 16S rDNA sequence of the isolate from the sample is more than 96% identical to *pseudomonas aeruginosa* strain NCIM2995. As a result, the isolated organism was verified to be *pseudomonas aeruginosa.*

Antibiotic susceptibility test:

Fig.8: Antibiotic susceptibility test

Antibiotics	Concentration	Zone of inhibition	Interpretation
Amoxiclav	30mcg	-	Resistant
Tobramycin	10mcg	30mm	Sensitive
Cefazolin (CZ)	30mcg	11mm	Resistant
Gentamicin (GEN)	10mcg	21mm	Sensitive
Cefuroxime	30mcg	-	Resistant
Amoxiline	30mcg	25mm	Sensitive
Cefotaxime	30mcg	30mm	Sensitive
Ciprofloxacin	5mcg	40mm	Sensitive
Tetracycline (TE)	30mcg	11mm	Resistant
Cefoperazone	30mcg	30mm	Sensitive
Ceftazidime	30 mcg	31mm	Sensitive
ofloxacin	5mcg	32mm	Sensitive

Table 12: Antibiotic susceptibility test

The antibiotic test was used to determine susceptibility profiles to sixteen antimicrobial compounds from six different classes. An antibiotic susceptibility test was performed using the Kirby-Bauer disc diffusion method [24]. The results are summarised in Table 12.

show that 09 *p. aeruginosa* isolates at different season were susceptible to 08 tested antibiotics tobramycin, gentamicin, amikacin, cefotaxime, ciprofloxacin, Cefoperazone, ceftazidime, ofloxacin and 4 were resistant to Cefazolin, cefuroxime, amoxilin, tetracycline. Previous investigations have indicated a widespread dispersion of antibiotic-resistant bacteria in surface and ground waters.[25][26] The findings of the current study are thus not unusual. However, there is concern regarding antibiotic resistance in the microbes found at the source.

Conclusion:

The aim of present study is To study the prevalence and drug resistance in *Pseudomonas aeruginosa* from Drinking Water Sources of Morbe Dam, Navi Mumbai. *Pseudomonas aeruginosa* were present in Morbe dam water samples collected in 3 seasons during the month of March 2020, July 2020, December 2020.isolated bacteria was confirmed by biochemical tests, 16s RNA sequencing, and antibiotic susceptibility test. This study demonstrated that isolated pseudomonas aeruginosa was responsive to antibiotics.

Tobramycin (TOB), Gentamicin (GEN), Cefuroxime (CXM), Amikacin (AX), Cefotaxime (CTX) and resistance toward Amoxiclav (AMC), Cefazolin (CZ), Cefuroxime (CXM), Tetracycline

(TE). So, study suggests that isolated organism was resistance towards four drugs, but it was not found in MDR.

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