Detection of Class 1 integron-*intI1* Associated with Neomycin and Ciprofloxacin Resistant Bacteriain Different Spots of Bay Receiving Sewage water

Saad Hameed Abood[1], Saif Talib Jasim[2], Zaynab Khorsheed Rasheed[3], Shadman Tariq Sadiq[4]

[1]Tutor, Biology Department, College of Science, AL-Iraqyia University. Baghdad, Iraq, [2]Asst. Department of Al Qaim Education, General Directorate of Education in Anbar, Ministry of Education, Anbar, Iraq, [3]Asst., Northern Technical University, Technical college Kirkuk, Kirkuk, Iraq, [4]Tutor, Biology Department, College of Science, Tikrit of University, Salahaddin, Iraq

Abstract

In this study, Class 1 Integron co associated with some antibiotics resistant bacteria was investigated from different spots on Izmir bay of Aegean sea as a first report about analysis and track of influences human and animal wastes on water environment in a period between July 2019 – January 2020.

Our results showed variations in Neomycin and Ciprofloxacin resistance bacteria, Neomycin resistance bacteria was detected in100 % of all sampling points ranged from 4.2×10^2 in (BOS) samples to 1.98×10 revealing wide distribution of Neomycin resistance bacteria in different spots on Izmir bay receiving sewage water.

Ciprofloxacin resistance bacteria founded in 25 % of collected samples it was only founded in (BS) sampling point, Class 1 integron investigation, showed wide range distribution during sampling campaign among different sampling points, the main results showed that 75 % of samples was positive to class 1 integron presence, it was founded in Balçova Ilica (BS), Melez (MS), Bornova (BOS) points with no appear in the fourth point Bostanli streams (BTS).

During the investigations we noticed that Class 1 integron elements was associated 100 % with Neomycin resistance bacteria in four sampling points (BS, MS, BOS & BTS) while showed weak association with Ciprofloxacin resistant bacteria.

Keywords: *Neomycin resistance bacteria, Class 1 integron, Izmir bay.*

Introduction

Integrons, especially class 1ıntegron is a platform that allow capturing, storage, cleavage and re-arrangement of many genes in the same frame calling gene cassettes,Class 1 ıntegron {intI1} possess genes encodes for producing of integrase factor. This element considers one of the most critical transportable genetic elementsdue to its role in the spreadıngof antıbiotic resıstance genes (ARGs) through horizontal gene transfer(HGT) between bacterial communities,facilitating their transfer into a wide range of pathogenic bacteriaand carry antibiotic resistance genes from one bacteria to another bacterial cellsimposing risks to public health(1-3).

Although Integrons similar to plasmids and transposons as a HGTelementbut different from theme by capability of capturing gene forming resistance gene cassettes incorporated into integron frame then enables

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Correspondence Author:
Shadman Tariq Sadiq
Tutor, Biology Department, College of Science, Tikrit of University, Salahaddin, Iraq
e-mail: shadmantsm@tu.edu.iq
Mobile No.: +9647517094645

integrons to mobiling across different microbial species making it to be predominantly associated with multi drug resistance-MDR gram-negative bacteria, also sometimes embedded in promiscuous transposons and plasmids(4-6).

Generally, class 1 integron composed of three main parts: an (intI1) gene encodes for integrase enzyme which enabling this platform to capture and expression of exogenous genes into second part *(attI)* called recombination site forming gene cassettes, third part is a promoter *(P_C)* catalyze inserted genes expression. (7-8)

There are many researchers reported that Class 1 integron exists in different environments with considerable sequence diversity Gillings *etal* 2008b also previously reported to be the most abundant intI in Wastewater treatment plants without completely elimination by different treatment processes. In addition, investigated in Chicken Meat (9), ready-to-eat foods in China (10), Clinical isolates (11-12), prevalence in pigs and pork (13), founded ın about of 81% of *Escherichia coli* isolates from waterfowls in China (14), **r**ecovered among fruits and vegetables (15)also detected in guts of *Salmo salar* fish farms (16)all these reports reveal the size of this element distribution.

The most interested point of class 1 integron is linking of this element not only withantibiotics resistance, also associated with heavy metals resistance genes making it responsible for disseminating of resistance from pathogenic microbes to environmental non-resistance microbes and spreading from its common origin gram-negative bacteria into gram-positive bacteria species(1-2; 7).

intI1 gene has a specificity to be a generic marker of anthropogenic produced pollution including high abundance in domestic animals and human's bacteria, a consequently high representation in streams, based on these, many researchers have used of intI1 analysis to track anımal & human influence on water environment(7).

Understanding the presence, origin and fate of these elements is important for the practical control of antibiotic resistance and for exploring how lateral gene transfer can seriously impact byhuman activities, also the association of this mobile gene element MGE with ARB can accelerate the dissemination of ARGs through HGT in the water environment making the problem of antibiotics resistance more sever(17).

However, due to limited data are available on the class 1 integron distribution and its association Ciprofloxacin-Neomycin Resistant bacteria in entire Turkey and no previous investigations about prevalence of class 1 integrons in Izmir baytherefore, we started screeningthis element presenceto determine the range of distribution from sewage water to Izmir bay and recording afirst documentation to serve in future works regarding to antibiotic-resistant bacteria (ARB) and ARGs associated with class 1 integron.

Material and Method

Study area and Samples processing: Izmir Bayfigure(1) (also Smyrna golf) is located on Aegean Sea in Izmir city between 38° 18' 00' - 38° 43' 00'' latitudes and 26° 23' 00' – 27° 11' 00'' longitudes. Its length 64 km by 32 km in breath. About 20 streams flowing into the inner part of the Izmir Bay. Samples collected from BalçovaIlica(BS), Melez (MS), Bornova (BOS)and Bostanli streams (BTS) as marked in figure(2-A, B,C,D).

Grab sampling method applied for collecting of samples in a period between July 2019 – March 2020 water samples were brought to the laboratory in a freezer in glass container and stored in the refrigerator at + 4°C for further investigations.

Antibiotics resistant bacteria (ARB) counting: Bacterial counting was performed to test phenotypic resistance of both Neomycin and Ciprofloxacin resistance. R2A agar + interested antibiotic solution and plate pouring method was applied to perform this step following standard protocol of Clinical and Laboratory Standard Institute - CLSI instructions(18-19).

Antibiotic solutions were Neomycin:10 µg and ciprofloxacin:5 µg. Both of them were sterilized by 0.22 µM Millipore filters and added to R2A agar before pouring.

DNA extraction and manipulation: Total DNA was extracted directly from water samples using commercial Kit *(NorGenbiotek™, Canada)* following the manufacturer's instructions. All DNA extracts were checked for its quality on 1 % of gel electrophoreses and Nanodrop™.

Primers & PCR reaction: The occurrences of class 1 integron in the water samples were first determined using convenient PCR, the PCR running conditions is presented in Table 1. Specific primers were ordered from *Genaid*^{TM}, all primers were analyzed by *BLAST program*, these quences and other details of the primers showed in Table 2.

PCR program was done as following steps: initial (ID) at 94◦C for 3 min, followed by 30 cycles of denaturing(D) at 94°C for 30 s,annealing(ANN)was 64°C for 1 min, and elongating(EL) at 72°C for 90 s, final elongation was at 72◦C for 5 min. All PCR products were run on the agarose gel electrophoresis again to see int1 bands. detection and characterization of class 1integron was performed as described previously by[20-21].

Fig. (1): Izmir bay marked with samples collecting spots

A **B**

C D

Fig. (2): Sampling spots on Izmir bay receiving Sewage water, A: Balçova Ilica stream, B: Melez stream, C: Bornova stream, C: Bostanlı streams.

Table (1): Primers used in the stud

Primers PCR steps	**aph (3`)-IIa (Neomycin)**	***qnrS* (Ciprofloxacin)**	**IntI1 Class 1 integron**
Initial denaturation	95˚C 2 Mn	95˚C 2 Mn	94˚C/3Mn
Denaturation	95˚C 30 S 30 cycle	95˚C 30 S 30 cycle	94˚C/30 s 30 cycle
Annealing	60 C 30 S 30 cycle	61 30 S 30 cycle	64˚C 1 Mn 30 cycle
Elongation	72 C 30S 30 cycle	72 30S 30 cycle	72˚C 90 S 30 cycle
Final elongation	72˚C 5 Mn	72˚C 5 Mn	72˚C 5 Mn

Table (2): PCR reaction steps and conditions

Resistance gene	**Primer name**	**Primer sequence**	**Amplicon size**	**Anealing C**	**Source**
Class 1 integron	Int I1	5’- GTTCGGTCAAGGTTCTG 3’ 5’- GCCAACTTTCAGCACATG 3’	923	50	(21)
Ciprofloxacin	qnrS	F: ATCAAGTGAGTAATCGTATGTACT R: CACCTCGACTTAAGTCTGAC	171 bp	61	(22)
Amikacin/Neomycin	aph(3`)-IIa	F : ATGATTGAACAAGATGGATTGC R: TCAGAAGAACTCGTCAAGAAGG	795bp	60	(23)

Results and Discussion

The presence of Neomycin and ciprofloxacin resistance bacteria with class 1 integron consider strong relationship for anthropogenic effects of antibiotic resistance bacteria on water environment especially natural water resource like our sampling spot in Izmir bay that receive sewage water contain different antibiotic resistance bacteria and transposable elements like class 1 integron.

As well as using of Neomycin in clinical it’s also used in veterınary field for the treatment of diseases in sheep, cattle, goats and swine, so, the presence of this antibiotic associated with class 1 integron consider marker for pollution of sewage water with animal related waste which increase the impact on normal flora of sea water. The same issue with Ciprofloxacin, a fluoroquinolone antibiotic used in clinical and animal like dogs and cats for control diseases, even its isolated from poultry products(24) may play a role as marker of pollution by animal products wastes that may discarded into some sewage that finally flowed into sea water by streams.

Our results showed variations in Neomycin and Ciprofloxacin resistance bacteria, Neomycin resistance bacteria was detected in100 % of all sampling points ranged from 4.2×10^2 in (BOS)samples to 1.98×10 revealing wide distribution of Neomycin resistance bacteria in different spots on Izmir bay receiving sewage water.

Ciprofloxacin resistance bacteria founded in 25 % of collected samples it was only founded in (BS) sampling point; this result is much lower thane neomycin resistance bacteria in our work erea may be due to using of Neomycin more thane ciprofloxacin espicially in veterinary uses.

In other work by (25)reported that all strains, except SI40 strain were resistant to Neomycin and other tested antibiotics for samples isolated from chicken broils.

As we explained until now there is no previous works on the same antibiotics resistance to make a compression but there are some works related to the same antibiotics in other project(24) founded that among all isolates tested 91.4% were phenotypically resistant to ciprofloxacin(26) Kuang etal 2018 reported that about 2.23 % of water samples was resistant to ciprofloxacin while no resistance record for isolates investigated by Abbasoglu and akcelik in the same work discussed above.

The resistance to Ciprofloxacin (fluoroquinolones) is mainly related to amino acids substitutions in the quinolone resistance determining region - QRDR in ciprofloxacin resistance gene (27)this case confirmed by(28)when they observed amino acid change that lead to ciprofloxacin resistance.

Regarding to genotypic resistance characterization our results not matched with Neomycin and Ciprofloxacin appeared phenotypic resistance this may due to inhibitors founded in sewage water samples.

Class 1 integron investigation, showed wide range distribution during sampling campaign among different sampling points, the main results showed that 75 % of samples was positive to class 1 integron presence, it was founded in Balçova Ilica (BS), Melez (MS), Bornova (BOS) points with no appear in the fourth point *Bostanli* streams (BTS) figure(3), these points may adjacency with some animal farms and mini livestock breeding houses that may discharge their waste to these streams, especially results not appeared at fourth sampling point (BST) for all sampling campaign.

During the investigations we noticed that Class 1 integron elements was associated 100 % with Neomycin resistance bacteria in four sampling points (BS, MS, BOS & BTS) while showed weak association with Ciprofloxacin resistant bacteria.

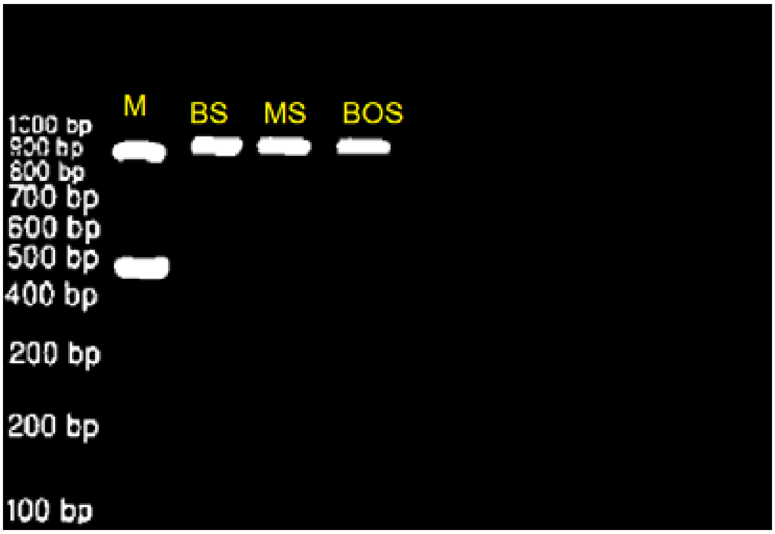

Fig. (3): PCR amplification products image on 1.5 % agarose gel electrophoresis for class 1 integron using specific primer for different sampling spots, M; DNA ladder (100 bp), BS: Balçova Ilica stream, MS: Melez stream, BOS Bornova stream (900-923 bp) prospectively.

The relationship of class 1 integron with Neomycin–Ciprofloxacin resistant bacteria and other antibiotics resistance discussed in many previous reports.[29] Li *etal* 2013 observed strong relationship of Class 1 integron and resistance to a variety of drugs like ciprofloxacin (both phenotypes and genotypic).among 176 isolates of *Klepsiella pneumoniae* class 1 integron positive isolates exhibited resistance to number of drugs as much as higher than those negative isolates that miss to integron also the extensively drug resistant isolates and including those located outside and within of frame was significantly higher among class 1 integron positive isolates.

The worst point of association between integron and antibiotic resistance is persistence in the polluted water even after treatment,[30] Wang *etal* 2019 demonstrated that the wastewater treatment systems could not completely able to remove the antibiotic resistance genes that associated with mobile genetic material like integrons, as a results probably be transferred into human forming great threats on public health.

Conclusion

The increasing amount of antibiotics using in clinical and veterinary field consider the basic reason for dissemination of antibiotics resistance, the worst point of this problem when disseminate by mobile genetic elements harbor many resistance genes cassette like class 1 integron.

The expanding of articles submissions concerned with class 1 integrons genes indicates that this situation is evolving and poses a potential clinical problem that could get more complex to be solved in advance.

In conclusion this study represents the first report showing screening of integron associated with some antibiotics resistance in Izmir bay, the investigations showed that the variations of Neomycin and Ciprofloxacin resistance bacteria, revealing wide distribution of Neomycin resistance bacteria in different spots on Izmir bay receiving sewage water.

Ciprofloxacin resistance bacteria was only founded in (BS) sampling point; this result is much lower thane neomycin resistance bacteria in our work erea may be due to using of Neomycin more thane ciprofloxacin espicially in veterinary uses.

wide range distribution of Class 1 integron in Balçova Ilica (BS), Melez (MS), Bornova (BOS) pointsmay adjacency with some animal farms and mini livestock breeding houses that may discharge their waste to these streams, especially results not appeared at fourth sampling point (BST) for all sampling campaign.

Finally, tested parameters may be changed with different seasons or in case establishment of waste water treatment systems before discharged into this bay.

Ethical Clearance: Not required

Conflict of Interest: No conflict of interest.

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