ORIGINAL ARTICLE

The Effect of Titanium Dioxide Nanoparticle on some properties of Resistance Gram Negative Bacteria

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ABSTRACT

In this study, the bacteriostatic,bactericidal, ant-biofilm, and molecular effectsof titanium dioxide nanoparticles (TiO_2NP)against multidrug resistance (MDR),Extended Spectrum βeta-Lactamase (ESBL)and*AmpC* producer pathogenic isolates from different clinical specimens was assessed. Based on the results and given the(Minimum Inhibitory Concentration), MBC (Minimum Bactericidal Concentration), of titanium dioxide (10 nm size) were 0.488 µg/ml and 0.97 µg/ml and for Tio2NP (100 nm size) were 7.90µg/ml and 15.80for resistance pathogenicE.coli isolates.On the other hand, the inhibition zoneof incubated isolates in 10nm and 100 nm nanoparticles increased non significantly as compared with control and small size was more effective than large size, and there is no resistance against nanoparticles in drugs resistance pathogenic bacteriaWe showed that bacterial biofilm growth was reduced at MICconcentrations of TiO2NPs compared with another test without the NPs. FurthermorePolymerase chain reaction amplification of AmpCscreened and sequence alignments, analyses performed using the Basic Local Alignment Search Tool (BLAST) online program that compare the results of traces nitrogen bases of the gene within the National Center for Biotechnology Information (NCBI) the result showed high similarities (98%)with the AmpC for the E coli isolate (control), This study provides that substitution in the nitrogen basethymine to guanine in sequence 17 and guanine to adenine in sequence 96 and 113was detected after incubation in titanium dioxide 10nm and substitution guanine to adenine in nitrogen base 92 and cytosine to thymine in nitrogen base 184 after incubation in Tio2NP 100nmConclusion: Antibiotics resistance beta-lactam gram-negative bacteria isolated from clinical samples has been detectedin hospitals. Since this bacterium is highly resistant to drugs, several new methods have been investigated to fight against it. Applying nanoparticles of titanium dioxide as a strongantimicrobialcompound,is one of the most effective ways to solve the bacterialmultidrug resistance increase problem.

Keywords:MDR,*AmpC*, Gram negative bacteria,Titanium dioxide nanoparticle

INTRODUCTION

The challenge of antibiotic resistance stimulates the researchers to explore the new agents that can effectively inhibit microbial expansion. Now nanoparticles (NPs) have showed broad-spectrum antibacterial properties for both gram-negative and gram-positive bacteriaand can complement, support traditional antibiotics and They are highly promising and are of great interest because they could fill the gaps where antibiotics often fail[1].

The Titanium dioxide nanoparticle TiO2NPs is a white metal oxide nanoparticle have a wide spectrum of antimicrobial activity toward a wide-range of pathogenic bacteria upon illumination of light due to its photocatalytic properties, their toxicity, by visible lightinduced or near-UV, approved reactive oxygen species burst, then production of ROS in microbial cells(2, 3).Rajakumar *et al*(2012) reported that the TiO2 NPsis a preferred antibacterialto E. coli[4]. Hassan *et al*(2012) reported their antibacterialactivity against E.coli, Salmonella typhimurium and Klebsiella pneumonia[5]. Also Marciano *et al*(2009) studied the bactericidal activity of TiO2 NPs on *E. coli*[6].

The aims of this study were to explore the impact of two size TiO2 NPs on some properties of resistance gram negative bacteria isolated from different clinical sources

MATERIAL AND METHODS

Isolation and Identification of bacteria: This study included 180 clinical specimens(Blood, Urine, Burn and Wound) were collected from patients that admitted to the "Teaching Hospital" in Erbil, Iraq. All specimens were subjected to standard bacteriological processes of culturing on MacConkey's and blood agar plates for 24–48 hoursfor isolation and purification at 37°C (7). Pure colony of each type of bacterial isolates according to cultural characteristics were identified, microscopic examination1, some biochemical tests, API-20E and finally were confirmed by Vitek 2 compact system (Biomerieux).

Antibiogram: Testing of antibiotic susceptibility was performed by the Kirby Bauer method on Mueller-Hinton agar regarding to the Clinical and Laboratory Standards Institute (CLSI) procedure (8).Isolates were cultured on Muller-Hinton Agar plate thenampicillin(10)(AMP), amoxicillin-clavulanic acid (AMC), cefazolin (10) (CFZ), ceftazidime (30)(CAZ), ceftriaxone (10)(CRO), cefepime(30) (FEP), imipenem (10)(IPM), Erythromycin (15)(E) and Nitrofurantoin (300)(NF) disks (Himedia, Mumbai, India) were placed on the media in 20-30 mm with other disks. For 18-24 hours at 37°C the plates were incubated.

ESBLandAmpCscreening: Screening ESBL producer isolates was done by ESBL screening test by VITEK 2 system utilizes the growth response to ceftazidime, cefepime, and cefotaxime in mixing with or without clavulanic acid considered indicative of ESBL production. The ESBL producer isolates was inoculating on Mueller-Hinton agar and cefoxitin (30mg)disk and another containing 30 µg of cefoxitin and 400 µg of boronic acid

was placed on the agar plates, incubated overnight at 35°C. The bacteria that showed 5 mm or larger zone surround the disk containing cefoxitin and boronic acid compared to that containing cefoxitin was supposed as *AmpC* producer[9].

Prepare stock solutions of nanoparticles: The TiO2 NPs was obtained from the M K Impex Corp company CANADA in the form of plastic explosive 25 g capacity measuring (10 and 100)nm has been received in the form of nanopowder, and an area of superficial grave of 225 g/m2, the degree of purity of 99%, moisture 0.48%.TiO2NPs measuring (100)nm and degree of purity of 99%, an area of superficial grave of 16.8 g/m2, density 3.9 g/ml at temperature degree 20 °C.

Stock solution of NPs was prepared by added 1mg of NPs to 1ml of deionizer water and shake vigorously for 5 minutes to break point and have a homogeneous solution and then sterilized in autoclave at 121°C for 20 minutes and cooled in 25 °Cfor a final concentration of stocks 1000µg / mL[10].

The antimicrobial impact of Tio2NPs was tested by using the standard broth dilution method (CLSI). The MIC was calculated in nutrient broth using serial two-fold dilutions of (10nm and 100nm Tio2NPs) in concentrations ranging from 750 µg / mL to 0.030µg / mL with equally bacterial concentration (0.10 at 625 nm (1×108 CFU/ ml, 0.5 McFarland's standard). The positive control used in this analysis included a nutrient broth medium with bacterial concentrations measuredand negative control included only inoculated broth and 24 h and 37ºC incubation time and temperature respectively. The MIC was noted by the visual turbidity of the tubes both before and after incubation. Those tubes that display no noticeable growth of the bacteria seeded on nutrient agarplates that are not supplemented with Tio2NPs were incubated at 37ºC for 24 hrs. Whereas MBC was identified as the lowest antibacterial agent concentration, which reduces the viability of theinitial bacterial inoculum by 99.9%, due to the presence or lack of bacterial growth in agar plates.

Effect of Tio_2NPs on biofilms formation in ESBL and*AmpC*producer: A loopful of test isolate was inoculated with 1 per cent glucose in test tubes in 10 mL of trypticase soy broth (TSB) and then incubated at 37oC for 24 h. Tubes were decanted and washed with purified water after incubation, dried and coated for 30 minutes with crystal violet (0.1 percent). Excess stain was cleaned, reversed place dried tubes. The result for tube method was done regarding to the results of the control(TSB tube with crystal violet and without bacterial isolate and Tio2Nps) strains. When a visible film lined the wall and the bottom of the tube,biofilm formation was considered positive. The amount of biofilm formed was scored as (-)none, (+)weak, (++) moderate and (+++) strong. The trial was performed in triplicate.Data are documented and compared control[11].

DNA extraction and PCR: DNA was extracted from isolates grown on agar medium using the DNA extraction kit (Promega) according to manufacture instructions.

The specific primers for amplifying the *AmpC* gene by PCR are shown in Table 1. The PCR products were analyzed by agarose gels electrophoresis (Figure 4 A and B)and then the PCR product which was equivalent to expected amplification size in each cluster sent to Immuno Gene Center (IGC) in Erbil.for analyzing via sequencing Figure (6).

Table 1: The primer sequences of the AmpC genes amplified by PCR

Primer name	Sequence(5' 3')	Expected amplicon size (bp)
AmpC-F	AATGGGTTTTCTAC GGTCTG	191
AmpC-R	GGGCAGCAAATGT GGAGCAA	191

RESULTS

Table2 show that from total 180 clinical specimens 141(**78.3**%) was culture positive most of them 42.2% was urine followed by wound, burn and blood 26.7 ,18.9 and 12.2 respectively.

Gram negative were isolated which based on cultural characteristics, microscopic examination1, some biochemical tests, API-20E and finally were confirmed by Vitek 2 compact system (Biomerieux). As showed in Table 3 that the vast majority of the isolates were in urine (55.6%) in which (38.3%) was *E coli* followed by *K. pneumonia* (25.3%).

The data mentioned in Figure (1) and (2) explained bacterial antibiogram of most widely used antibiotics. Among the 162 isolates tested, 72 were susceptible to all antibiotics tested, including third-generation cephalosporins. Of the remaining 90 isolatesall (100%) were resistance to ampicillin followed by ceftriaxone, cefepime and Nitrofurantoin. Antibiotic susceptibility of 37*E. coli* isolates was evaluated for tested antimicrobials. The majority of *E. coli* isolates were resistant to most of the agents, including amoxicillin and Nitrofurantoin (100%), amoxicillin-clavulanic acid (70%), cefazolin and Erythromycin (87.5%), ceftazidime (90%), ceftriaxone (97.3%), cefepime (86.4%) andimipenem (40%).

Because 90/162 (55.6%) of isolates showed resistance to most of the antibiotic agents tested, in addition, multidrug resistance (MDR) was found in some isolates. Therefore, screening ESBLs producer isolates was determined phenotypically by VITEK 2,only 11(12.2%) *E.coli*,8(8.9%) *K.pnumonia*and 4 (3.3%) *P. aeroginosa*were ESBL producer isolates *AmpC* β-lactamase production was confirmed in only 3 (27.3%) of 11ESBL producer *E.coli*isolates and in the remaining (n = 8), it was not detectable.

About(27.3%)*E coli* isolates wereESBL and *AmpC*producer , therefore they selected to carry out the antimicrobial activity of TiO2NPssize 10nmand 100nm against them.When bacterial growth at different concentrations of TiO2NPs was assessed after 24 hours,the MIC and MBC of Tio2NP (10 nm size) were 0.488µg/ml and 0.97µg/ml and the MIC and MBC of Tio2NP (100 nm size) were **7.90**µg/mland **15.80**µg/mlfor all of clinical isolated *E.coli*Table (4).

The visible bacterial growth on nutrient agar plates was observed at 0.488 µg/mland 7.90µg/mlfor 10nm and 100nm respectively,thisvisible bacterial growthand positive control growth used in this study (that contained nutrient broth medium with tested bacterial concentrations)was used in all

following tests to determinate the effect of two size of Tio2NP on some properties of MDR, ESBL *AmpCE.coli* producer.

Comparison the inhibition zone using GraphPad Prism 6 as in Figure 3 showen thatinhibtion zone of some antibiotic against the isolates was increased non siginficantlly (P value 1)after incubation Tio_2NPas compared with control .The results revealed that the antibacterial effect recorded for small sizewas more effective than large size , the amoxicillin-clavulanic acid, ceftriaxone and Erythromycin zone interpretation were changed but not significantly. The feature of TiO2 NPs can be used to protect medical devices such as catheters in order to prevent opportunistic pathogenic infection[12].

The pathogenicity , chances of opportunistic infections and hard to control is rised in biofilm making bacterium. The ability of biofilm forming bacteria to show resistance to antibiotics. The ability of a bacterium to form a biofilm enhanced them to become common contaminant in clinicaltools like catheters. The immune suppresed host is very prone to the infections by such pathogen.TiO2 NPs moving beyond viability and growth; a functional assessment performed examining specific function. A comparative study was done to study the biofilm formation in under study bacteria control and incubation in 10nm and 100nm size Tio2NP. As mentioned in Table 5, biofilm formation was reduced in the presence of 10nm TiO2NP .

Phenotypicscreening ESBL *AmpC* beta-lactamase producer *E.coli*isolates was confirmed molecularly in order to detected ESBL enzymes coded by *AmpC* geneand all*E.coli* isolates were carries*AmpC* gene.A comparative study was done to screen the moleculer effect of Tio_2NPon *AmpC* genein under study isolatescontrol and ncubation in each10nm and 100nm size Tio2NPas in Figure (4 A and B) . The PCR product which was equivalent to expected amplification size was send to Immuno Gene Center (IGC) in Erbil for analyzing via sequencing.

The PCR products of *AmpC* gene were used as a template to analyze and determine the sequence of nitrogenous bases and confirm gene diagnosis in under study *E.coli* isolates. Sequence alignments and analyses were done using the Basic Local Alignment Search Tool (BLAST) online program that compare the results of traces nitrogen bases of the gene within the National Center for Biotechnology Information (NCBI)).The result showed high similarities (98%)with the gene *AmpC* for the E coli isolate under study, as shown in Figure (5) This study provides substitution in the nitrogen base thymine to guanine in sequence 17 and guanine to adenine in sequence 96 and 113 detected after incubation in Tio2NP 10nm substitution guanine to adenine in nitrogen base 92 and cytosine to thymine in nitrogen base 155 after incubation in Tio2NP 100nm.

Table 2: Number and percentage of clinical specimens

types of specimen	Total Specimens	Positive cultured specimens		negative cultured specimens	
		N	%	N	%
Blood	22(12.2)	9	40.9	13	59.1
Urine	76(42.2)	61	80.3	15	19.7
Burn	34(18.9)	31	91.2	3	8.8
wound	48(26.7)	40	83.3	8	16.7
Total	180	141	78.3	39	21.7

Table 3: Number and percentage of each bacterial isolates in each clinical specimens

Types ofbacterial isolates	Total isolates	Blood	urine	Burn	Wound
	N(%)	N(%)	N(%)	N(%)	N(%)
E. coli	62(38.3)	3(4.8)	46(74.2)	5(8.1)	8(12.9)
K. pneumonia	41(25.3)	1(2.4)	22(53.7)	10(24.4)	8(19.5)
Ps. aeruginosa	30(18.5)	4(13.3)	2(6.7)	15(50)	9(30)
baumannii	12(7.4)	1(8.3)	5(41.7)	4(33.3)	2(16.7)
p. mirabilis	17(10.5)	0(0)	15(88.2)	1(5.9)	1(5.9)
Total	162	9(5.5	90(55.6)	35(21.6)	28(17.3)

Table 4. The MIC and MBC of Tio2NPon bacterialisolates

***E. coli* under study**	**10 nm nanoparticles of Tio2NP (µg/ml)**		**100 nm nanoparticles of Tio2NP(µg/ml)**	
	MIC	**MBC**	**MIC**	**MBC**
1	0.488	0.97	7.90	15.80
2	1.95	1.95	15.80	15.80
3	0.976	0.97	15.80	31.61

Table 5:Anti-biofilm effect of TiO_2NP size 10 (0.488 µg/ml)and 100nm (7.90µg/ml) onbacterial isolates

***E. coli* under study**	**Biofilm formation(control)**	**Biofilm formation(after incubation in Tio_2NP)**	
		10nm	**100nm**
1	**+++**	**-**	**+++**
2	**+++**	**++**	**+++**
3	**++**	**+**	**+**

Representation: (-) Non biofilm formation, Few (+) (++) moderate (+++) strong

Figure 1: The antibiotic resistance pattren of pathogenic bacterial isolates

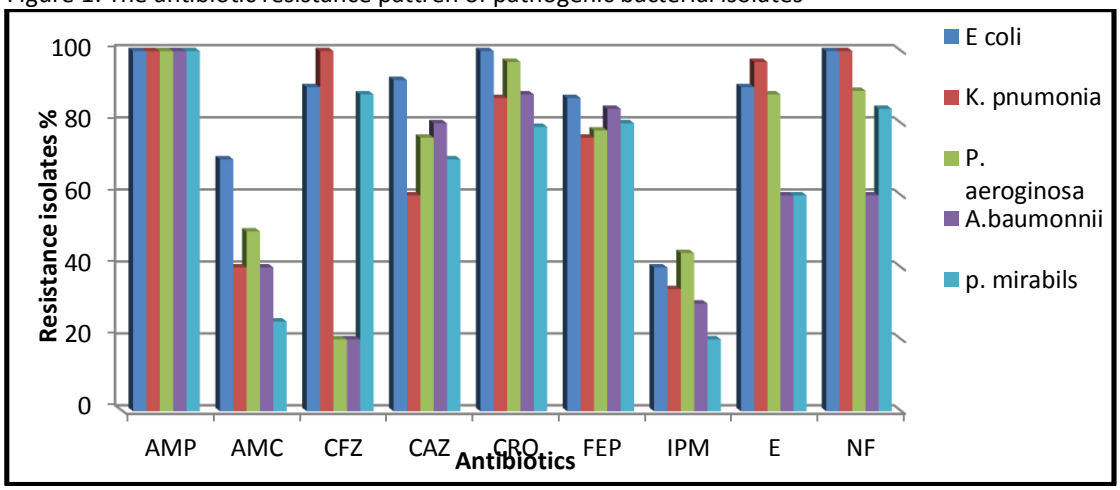


Figure 2 :Phenotypic screening ESBL and AmpC β-lactamaseproducing isolates

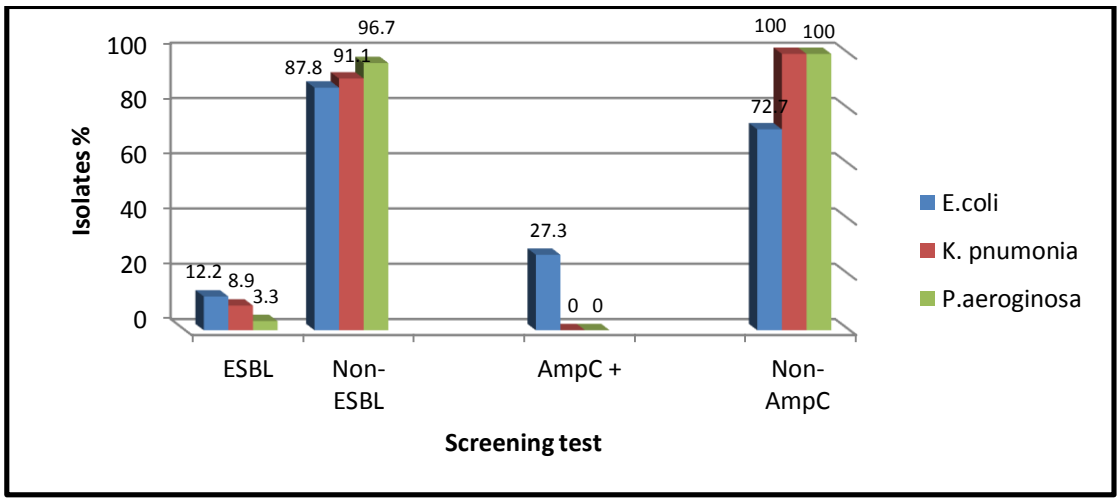


Figure3:The antibacterial activity of TiO2NP size 10 (0.488µg/ml)and 100nm (7.90 µg/ml) on bacterial isolates

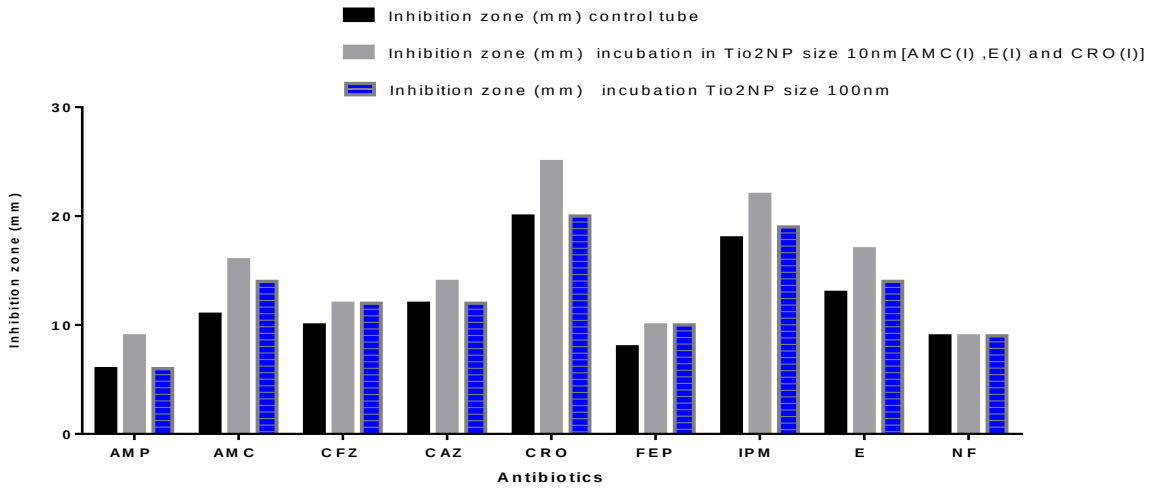
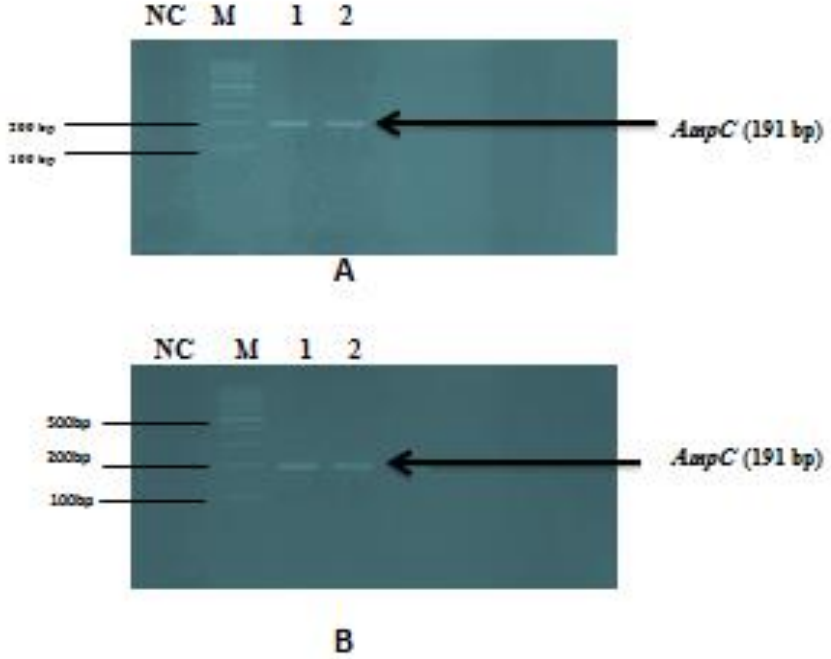


Figure 4: PCR amplification of AmpC Lane NC = Negative control, Lane M = 100 bp DNA marker, Lanes 1 and 2 = Clinical E coli isolates expressing AmpC



A-Control Isolates (without Tio2NP)
B-Isolates after incubation in Tio2NP size 10nm (0.488 µg/ml).

Escherichia coli strain 1919D62 chromosome, complete genome

259 bits(140) 2e-65 145/148(98%) 0/148(0%) Plus/Plus

```
Query  6        GCTGATTGGTGTCATTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCG  65
               ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4164503  GCTGATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCG  4164562

Query  66       GGCCGGTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCGMCTTATTAATT  125
               ||||| |||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  4164563  GGCCGTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCGCCTTATTAATT  4164622

Query  126      ACCGCCTCTTGCTCCACATTTGCTGCCC  153
               ||||||||||||||||||||||||||||
Sbjct  4164623  ACCGCCTCTTGCTCCACATTTGCTGCCC  4164650
```

A

Escherichia coli str. K-12 substr. MG1655, complete genome
257 bits(139) 4e-66 145/148(98%) 0/148(0%) Plus/Minus

```
Query 14   CGCGGATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGC 73
           ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct4379034CGCTGATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGC
Query   74 GGGCCGTTTTGTATGGAAACCAAACCCTATGTTCAAAACAACGCTCTGCGCCTTATTAAT 133
           |||||||||||||||||||||| |||||||||||||||| ||||||||||||||||||||
Sbjct4378974GGGCCGTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCGCCTTATTAAT
Query134  TACCGCCTCTTGCTCCACATTTGCTGCC 161
           ||||||||||||||||||||||||||||
Sbjct4378914TACCGCCTCTTGCTCCACATTTGCTGCC 4378887
```

B

Escherichia coli strain BCE049 chromosome, complete genome
95.3 bits(51) 3e-16 56/58(97%) 2/58(3%) Plus/Minus

Query 14 GATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGC 73
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 4897362 GATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGC 4897303
Query 74 CGTTTTGTATGGAAACCAAACCTTATGCTCAAAACGACGCTCTGCACCTTATTAATTACC 133
|||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct 4897302 CGTTTTGTATGGAAACCAGACCTTATGCTCAAAACGACGCTCTGCACCTTATTAATTACC 4897243
Query 135 GCCTCTTGCTCCACATTTGCTGCC 158
|||||||||||||||||||| |||
Sbjct 4897242 GCCTCTTGCTCCACATTTGCCGCC 4897219

C

Figure (5) AmpC gene sequence in E.coli isolate **A:** Control isolates
B: Substitution in the nitrogen basethymine to guanine in sequence 17 and guanine to adenine in sequence 96 and 113 detected after incubation in Tio2NP 10nm(0.488µg/ml)
C: Substitution guanine to adenine in nitrogen base 92 and cytosine to thymine in nitrogen base 155 after incubation in Tio2NP 100nm (7.90µg/ml).

DISCUSSION

All bacteria, particularly the gram-negative bacteria, can easily develop resistance to multiple antibiotics because of several resistance mechanisms to antimicrobial agents such as production of ESBL that makes the bacteria resistant to beta lactam antibiotics and also prone to developing resistance to other antibiotic classes[13].Accordingly, the findings of resistance to antibiotics was observed in previous studies, reported by[14] in Iran[15] in Mosul, Iraq[16] in Libya and[17] in Denmark.

A wide range of anti-infective agents have been used to well controlled the common infectious diseases, but nowadays, due to the huge use of these drugs, resistant strains have introduced. This reality and the belief in developing current treatments force the medical communityto try to search for new methods of combating infections[18].Nanotechnology has a range of functional roles to playin this field, from drug delivery through to diagnosis[19].Advances in nanoparticular systems for the diagnosis and treatment of infectious diseases are a promising field of researchwith major consequences for the treatment of bacterial infections, especially in the battle against multidrug-resistant strains and bacterial biofilms (20). Today, many of studies have been done to investigate the efficacy of antimicrobial and anti-biofilm of nanoparticles against the pathogens as drug resistance bacteria,the powerful broad-spectrum antibacterial activity of Tio2NP against pathogenic bacteria was recorded at500, 250, 125, 62.5, and 31.25 µg/ml[21]. A study showed the ability of nanoparticles TiO2 to inhibition of the layer Biofilm to all isolates of bacteria at concentrations (1, 1.5 µg/ml)[22].In further nanoparticles can be used for the coating medical devices in order to control the concerned bacterial infections. The antibiofilm activity of the TiO2 nanoparticles is important rather than antibacterial activity, because pathogenic bacteria grow in/on medical devices by producing biofilm. Biofilm existence of bacterial colonies indicates stronger tolerance to normal antibiotics and disinfectants used in hospitals to cleanmedical instruments such as catheters, kidney tubing, certain urinary tract diseases and inadequate roles such as biomedical applications[12]..

The findings of the present study revealed the prevalence of ESBL- and AmpC-producing*E. coli*to be 62.7% and 13.6%, respectively.The change in the nitrogen base pairing properties could be one of the possible causes of DNA sequence variations due to incubation of bacteria in nanoparticles which during the replication can lead to change the sequences in daughter strands. Variations in the DNA sequence may also be a cause for growth inhibition and cellcycle through mutations, accompanied through changes in gene expression correlated with growth and cell cycle regulation (23).A study concluded that metal oxide nanoparticles may interfere with the transcription and translation[24]Also Li *et al* stated that silver nanoparticles are influence on the DNA twisting, thus inhibit the replication and cell proliferation[25]. The findings of a study showed that silver and copper oxide nanoparticles not only prevent bacterial growth,but also alter the genomic DNA sequences and induce genetic variations between control and the samples being treated[26].

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