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RESEARCH ARTICLE

PREPARATION OF HYBRID NANO-CIPROFLOXACIN AND DETERMINATION ITS INHIBITION ACTIVITY AGAINST *E. COLI* ISOLATED FROM URINARY TRACT INFECTION PATIENTS IN HOLY KARBALA CITY

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ABSTRACT

Background: Urinary tract infections are the most common infections after upper respiratory tract infections *E. coli* caused 20% of urinary tract infection and it was responsible for 1/3 of the infections. Nanoparticles (NPs) in general have many properties that are different from those of traditionally used materials. They have dimensions typically below 100 nm, which allows them to reach specific sites inside the body and even to be permeable to tissues and cells. Therefore, they can deliver the drugs in active forms at sites that conventional drugs may not reach by themselves and thus minimize the undesirable side effects. Layered double hydroxides (LDHs) as one of the inorganic delivery nanoparticles have recently been receiving considerable attentions. Ciprofloxacin is a broad-spectrum antibiotic of the fluoroquinolone class. It is active against both Gram-positive and Gram-negative bacteria and it is recognized as one of the most effective antibiotics of the quinolone drug class and has been used for the treatment of urinary tract infections.

Aims and objectives: The aims of present study were to prepare hybrid nanoan-ciprofloxacin and characterize by spectroscopic methods, and determine its inhibitory activity against the *E. coli* isolates caused the urinary tract infection in this study.

Materials and Methods: In this study urine samples from 75 patients clinically diagnosed with urinary tract infection were collected into sterile screw capped container and used for culture, Gram staining, API E20, antibiotic sensitivity test and singleplex PCR methods. PCR was performed with primer pair targeted to the *PhoA* gene of *E. coli*. Hybrid nano-antibiotic represented by the Mg/Al-Ciprofloxacin-LDH was prepared by using the direct ion exchange method between the free ciprofloxacin antibiotic and Mg/Al-LDH carrier, and then the hybrid nano-ciprofloxacin was characterized by spectroscopic methods include FTIR, XRD and AFM.

Results: Out of 75 samples, 27(36%) samples have been detected for *E. coli* depending on the morphologic and biochemical characteristics of this bacterium on the nutrient agar, MacConky agar, blood base agar and EMB agar, as well as Gram stain and API E20. The antibiotic sensitivity test was showed different sensitivity patterns of *E. coli* isolates against the different types of antibiotics varied from marked sensitive pattern against imipenem and meropenem, to moderate sensitive pattern against ciprofloxacin, norfloxacin, levofloxacin, nitrofurantoin and amikacin, in addition, to the marked resistance pattern toward ampicillin, ticarcillin, amoxicillin-clavanic acid and piperacillin. The singlplex PCR positive samples were identified by presence of ~903bp amplicon of the *PhoA* gene. The results of FTIR, XRD and AFM methods of nano-ciprofloxacin characterization indicted that this prepared nano-antibiotic was hybrid nano-ciprofloxacin (Mg/Al-Ciprofloxacin-LDH). The results of inhibition activity of both free ciprofloxacin and hybrid nano-ciprofloxacin showed that these two forms of ciprofloxacin revealed highest inhibition activity against two *E. coli* isolates (IS8 and IS10) with diameter of inhibition zone 40 and 42 mm, respectively. Whereas the *E. coli* isolates IS1and IS2 were the least affected isolates by the inhibition activity of these two forms of cirofloxacin with diameter of inhibition zone 38 and 35 mm, respectively.

Conclusion: Using of hybrid nano-antibiotics resulting from loaded of different antibiotics on LDHs carrier could be represented a promising future tool to treat and control urinary tract infection and other different bacterial infections.

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INTRODUCTION

Urinary tract infections (UTIs) are an infection caused by the presence and growth of pathogen anywhere in the urinary tract including a kidney, ureter, bladder, and urethra (Czaja and Hooton, 2006). They are deemed the most prevalent infectious disease in that it has now touched the overall incidence of 18/1000 persons per year in the general population (Sohail and Khurshid, 2015). *Escherichia coli* is the most common cause of community and hospital acquired urinary tract infections (UTIs), it is responsible for >80% of all cases of UTI (Beatson *et al.,* 2015). *E. coli* isolates from uncomplicated community-acquired UTIs express a variety of virulence traits

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that promote the efficient colonization of the urinary tract (Toval *et al.,* 2013). Ciprofloxacin is an important and commonly used member of the fluoroquinolone group of antibiotics that are active against Gram-negative and Gram-positive organisms and they are used for the treatment of uncomplicated urinary tract infections. Ciprofloxacin inhibits DNA topoisomerase II and DNA topoisomerase IV activities, eventually leading to bacterial cell death (Goswami *et al.,* 2006) as shown in (figure 1) (Black *et al*., 2008).

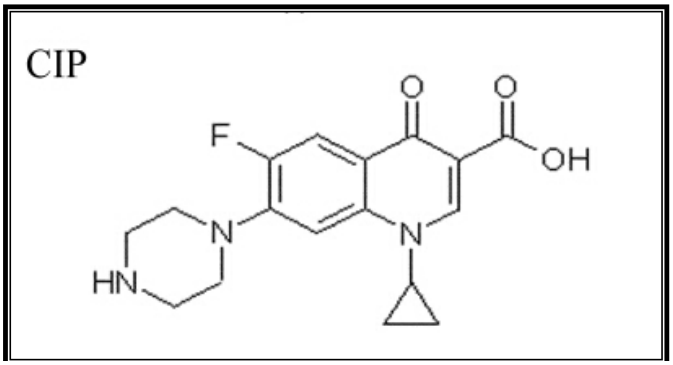

Figure 1. Chemical structure of ciprofloxacin (CIP) (Black *et al*., 2008)

Escherichia coli is a major global public health concern because it is becoming resistant to currently available antibiotics (Subash *et al.,* 2014). Across the United States and Canada, urinary tract isolates of *E. coli* from out patient clinics showed increased resistance to antibiotics (Zhanel *et al.,* 2000). This *E. coli* is named uropathogenic *E. coli* (UPEC) and have virulence properties that are associated with infection in the normal urinary tract including the expression of specific adhesions, toxins such as haemolysin, also the serum resistance, gelatinase production and P fimbriae which is considered as an essential virulence factor causing pyelonephritis (Ruiz *et al.,* 2002; Bahalo *et al.,* 2013).The increasing emergence of antimicrobial resistance warrants the need for the development of more effective broad-spectrum antibacterial agents. Therefore, nanotechnology advances could restore and rescue the currently available in-hand antibiotics by focusing on improved delivery methods and by localizing release to reduce adverse effects (Bell *et al.,* 2013). Modern drug delivery techniques operate on the highly beneficial principle of site-specific or targeted therapy. This area of research has been revolutionized by the development of nanotechnology. The use of nanoparticles in various medical applications has allowed for huge advances in cancer treatment, drug therapy and various applications related to visualization, sensing and gene delivery (Shrivastava *et al.,* 2007; Duncan and Gaspar, 2011). Furthermore, nanoparticles have numerous favorable properties, which include the ability to self-assemble, high stability in biological systems, specificity with regard to tissue targeting, the ability to encapsulate drugs, reduce the toxicity and side effects of drugs to host cell and providing image contrast for visualization purposes (De Jong and Borm, 2008; Zhao and Castranova, 2011). Layered double hydroxide (LDH) nanomaterial has emerged as a novel delivery agent for biomedical applications (Kim *et al.,* 2014). There has been remarkable development in the field to design controlled drug release by using LDH nanomaterials (Wang *et al.,* 2012; Zhang *et al.,* 2014; Barahuie *et al.,* 2014; Ariga *et al.,* 2014) as show in (Figure 2) (Bi *et al*., 2014). The use of LDH as a drug delivery system has been shown to increase biocompatibility, bioavailability (Kankala *et al.,* 2015) and solubility of hydrophobic drugs (Zhang *et al.,* 2008; Miao *et al.,* 2012; De Leon *et al.,* 2012; Kuo *et al.,* 2015; Kuthati *et al.,* 2015).

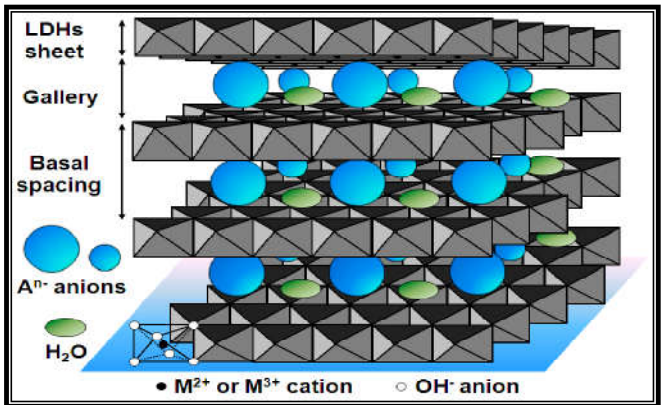

Figure 2. The structure of layered double hydroxides (LDHs) (Bi *et al*., 2014)

MATERIALS AND METHODS

Samples collection

During the period of study, 75 urine samples were collected from patients clinically diagnosed with urinary tract infection in Medical Al-Imam Al-Hussein City Hospital and Teaching childrens's Hospital, as well as Public Health Laboratory in Holy Karbala City. Patients' ages ranged from 10 to 30 years of age. This study was carried out after obtaining the approval from the Institute of Genetic Engineering and Biotechnology for Post Graduate Studies/ Baghdad University and Ministry of Health/ Iraq. Urine sample was obtained from patients of urinary tract infection and directly cultured on nutrient agar, MacConky agar, blood base agar and EMB agar (Merck, Germany) at 37°C for 48 hours under aerobic condition as deferential and enrichment media for *E. coli* from other *Enterobacteriaceae* members, then the Gram stains and API 20E test were performed. In addition, the antibiotic susceptibility test was carried out for *E coli* isolates using Kirby-Bauer disk diffusion method on nutrient agar according to clinical and laboratory standards institute (CLSI) guidelines.

DNA extraction

Genomic DNA was extracted from the *E. coli* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modification. Briefly, 1 ml of an overnight *E. coli* culture grown at 37°C in brain heart infusion broth (Sigma, USA) was transferred to a 1.5 ml microcentrifuge tube. The microcentrifuge tube was centrifuged at 14,000 rpm for 3 minutes to pellet the cells and the supernatant was removed. 600 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added and gently pipets until the cells are resuspended. The microcentrifuge tube was incubated in water bath at 80°C for 5 minutes to lyse the cells; then cool to room temperature. 3 µl of RNase solution (wizard genomic DNA purification kit) was added to the cell lysate and the microcentrifuge tube was inverted for 5 times to mix. The microcentrifuge tube was incubated at 37°C for 60 minutes and cool to room temperature. 200 µl of protein precipitation solution (wizard genomic DNA purification kit) was added to

the RNase-treated cell lysate and vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate. The microcentrifuge tube was incubated on ice for 5 minutes and centrifuged at 14,000 rpm for 5 minutes. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600 µl of room temperature isopropanol. The microcentrifuge tube was gently mixed by inversion until the thread-like strands of DNA form a visible mass and centrifuged at 14,000 rpm for 5 minutes. The supernatant was carefully poured off and the microcentrifuge tube was drained on clean absorbent paper. 600 µl of room temperature 70% ethanol was added and then the microcentrifuge tube was gently inverted several times to wash the DNA pellet. The microcentrifuge tube was centrifuged at 14,000 rpm for 2 minutes and the ethanol was carefully aspirated. The microcentrifuge tube was drained on clean absorbent paper and the pellet was allowed to air-dry for 15 minutes. 100 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added to the microcentrifuge tube and the DNA was rehydrated by incubating at 65°C for 1 hour. The solution was periodically mixed by gently tapping the microcentrifuge tube and the DNA sample was stored at -20°C until use.

DNA quantification

The extracted DNA from the *E. coli* isolates was quantified spectrophotometrically at O.D. 260/ 280 nm with ratios 1.5-1.6. The sensitivity of the PhoA-F and PhoA-R primers was evaluated by PCR amplification for serial diluted concentrations (10-100 ng) of purified genomic DNA isolated from *E. coli*.

Primers selection

The primers for *PhoA* gene of *E. coli* as the target gene for this study were selected according to (Kong *et al.,* 1999). This set of primers was designed based on the conserved region in *E. coli*, primers were synthesized by Alpha DNA, Kanda. The primers sequence of *PhoA* gene and their size of product are shown in (Table 1).

Table 1. The primers sequence of *PhoA* gene of *E. coli* and their product size

Name of Primer	Sequence of Primer (5'-3')	Size of Product
PhoA-F	GTGACAAAAGCCCGGACA CCATAAATGCCT	~903
PhoA-R	TACACTGTCATTACGTTGC GGATTTGGCGT	

PCR Master Mix

The PCR reactions for detection of *PhoA* gene of *E. coli* were performed in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 µl of GoTaq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 2.5 µl of 20 pmol PhoA-F primer and 2.5 µl of 20 pmol PhoA-R primer and 2 µl of the genomic DNA sample. The mixes were overlaid with 2 drops of mineral oil.

PCR program

PCR was carried out in a thermal cycler (Applied Biosystem 9902, Singapore) according to the PCR program described by (Kong *et al.,* 1999), with some modification. Briefly, the Amplification of *PhoA* gene of *E. coli* was carried out with initial denaturation at 95°C for 6 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for PhoA-F and PhoA-R primers for 90 seconds, and extension at 72°C for 2 minutes. The thermal cycles were terminated by a final extension for 10 minutes at 72°C.

PCR products analysis

The analysis of PCR products of *PhoA* gene of *E. coli* was performed on 1% agarose gel. The 1 kb DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 45 minutes at room temperature. The PCR products were stained with ethidium bromide and visualized by an image analyzer (ChemiImager 5500, Alpha Innotech, USA).

Preparation of hybrid nano-ciprofloxacin:

(i)- Preparation of magnesium/aluminum-layered duple hydroxide (Mg/Al-LDH) carrier

The magnesium nitrate/aluminum nitrate/layered duple hydroxide carrier that used in present study was prepared according to the method that described by Klemkaite *et al.,* (2011) as the following steps:

a- Preparation of 0.5 M magnesium nitrate (Mg $(NO_3)2.6H_2O$) solution

This solution was prepared by dissolving 32.05 gm of magnesium nitrate in 150 ml of deionized distilled water, and then the volume was completed up to 250 ml by deionized distilled water.

b- Preparation of 0.125 M aluminum nitrate ($Al(NO_3)3.9H_2O$) solution

This solution was prepared by dissolving 11.72 gm of aluminum nitrate in 150 ml of deionized distilled water, and then the volume was completed up to 250 ml by deionized distilled water.

c- Preparation of 2 M sodium hydroxide (NaOH) solution

This solution was prepared by dissolving 4 gm of sodium hydroxide in 25 ml of deionized distilled water, and then the volume was completed up to 50 ml by deionized distilled water.

(ii)- Preparation of carrier

The magnesium nitrate/aluminum nitrate/layered duple hydroxide (Mg/Al-LDH) carrier was prepared using co-precipitation method with some modification as fowling, the magnesium nitrate solution was mixed with the aluminum nitrate solution, which has been deposited at pH 10.5 by adding drop by drop of 2M sodium hydroxide solution with continued mixing by magnetic stirrer for 2 hours. The mixture was placed in the incubator at 70 °C for 18 hours. The precipitate was separated by centrifugation at 5000 rpm for 20

minutes.The precipitate was washed with dionized distilled water for several times, and then dried at 60 °C. The dried precipitate was grinded to obtain a fine powder, and then stored in the refrigerator until use.

(iii)- Preparation of antibiotic solutions

The antibiotics that used in present study were prepared according to method that described by Bashi *et al.* (2013) as the following steps:

a- Preparation of ciprofloxacin solution

This solution was prepared by dissolving 1.2 gm of ciprofloxacin in 25 ml of deionized distilled water, and then the volume was completed up to 50 ml by deionized distilled water.

b- Preparation of hybrid nanoantibiotics

The hybrid nanociprofloxacin was prepared from magnesium/aluminum-layerd duple hydroxide with ciprofloxacin antibiotics by direct ion exchange method according to method that described by Bashi *et al.* (2013) with some modification as following, fifty ml of ciprofloxacin was separately added to 50 ml of layered duple hydroxide solution (This solution was prepared by dissolving 1 gm of layered duple hydroxide precipitate in 50 ml of deionized distilled water). The mixture was stirred by magnetic stirrer at room temperature for 24 hours, and the mixture was placed in the incubator at 40 °C for 18 hours. The sediment was separated by centrifuge at 5000 rpm for 20 minutes. The sediment was washed with deionized distilled water for several times, and was dried at 50 °C. The sediment was grinded and stored until use.

Characterization of synthesized hybrid nano-ciprofloxacin

The hybrid nanociprofloxacin of present study were characterized by using several methods according to (Lim and Isshiki, 2005) and (Mir *et al.,* 2012), as following:

(i)- Fourier transform infrared spectroscopy (FTIR)

The infrared spectrum study for each of hybrid nanociprofloxacin and ciprofloxacin free form, as well as the layerd duple hydroxide (LDH) carrier of this study, was carried out using a disk for each antibiotic and the carrier were they mixed with the potassium bromide (KBr) after grinding well, the infrared spectrum was measured in wavelength numbers range from 400-4000 cm^{-1} and the obvious bands were set up with their wavelengths numbers and the most major bands were detected.

(ii)- X-ray diffraction spectrum (XRD)

The hybrid nanociprofloxacin of the present study were characterized using X-ray diffraction spectrum which explains the difference in the thickness of the layer before and after the overlapping process for each of ciprofloxacin and nalidixic acid by the (brak low $n\lambda = 2dSin\Theta$), to extract the value of the thickness of the layer (d) before and after the raid, n: represent the crystalline grade level at which the X-ray will be diffraction, λ: represent the wavelength used of X-rays, Θ: represent the diffraction X-ray angle, and d: represent the crystalline vertical distance between the two levels parallel.

(iii)- Atomic force microscope (AFM)

The hybrid nanociprofloxacin of this study were also characterized by using of atomic force microscope (AFM) to measure the diameters, sizes and aggregation of the nanoparticles. The samples of hybrid nanociprofloxacin were sent to Prof. Dr. Abdul-Karim Al-Samarrai/ Department of Chemistry/ College of Science/ University of Baghdad for this purpose.

(iv)- Precise analysis of C, H and N elements

The rates of C, H and N elements in the free form of ciprofloxacin antibiotics, and in the hybrid nanoantibiotics of ciprofloxacin was estimated to determine the antibiotic ratio inside the LDH carrier.

Detection of inhibitory activity of free and hybrid nano-ciprofloxacin against *E. coli*

The inhibitory activity of free form of the ciprofloxacin and hybrid nanociprofloxacin was detected against *E. coli* isolates of the present study, as following:

(i)- Preparation of bacterial inoculum

The bacterial inoculum was prepared by transfer 5 colonies of *E. coli* isolate grown on nutrient agar by sterile loop into a tube containing 5 ml of sterilize nutrient broth, and incubated at 37 °C for 24 hours, then appropriate amount of bacterial inoculum was transferred into tube containing normal saline solution to gate a growth turbidity equal to standard solution turbidity (McFarland tube No. 0.5), which gives an approximate cells number (1.5×108) colony formation units/ ml.

(ii)- Identification of inhibitory activity

The inhibitory activity of free form and hybrid nanociprofloxacin (Mg/Al-CIP-LDH), was identified against the *E. coli* isolates of present study that were sensitive for ciprofloxacin antibiotics which were used with different gradient concentrations include 0.001, 0.01, 0.05, 0.1, 0.25, 0.5 and 1 mg/ml according to the diffusion method in agar by drilling (Egorove, 1985), as follow, 100 µl of bacterial inoculum was withdrawal and spread on plates of Muller Hinton agar by L-Shape spreader and, then the Petri dishes or plates were left in the refrigerator for 1 hour. The wells were performed in the inoculated Muller Hinton agar plates by using the cork piercing with a diameter of 5 mm for each well with equal distance among the wells. The free form and hybrid nanociprofloxacin were diluted by using dimethylsulfoxide (DMSO) to get different gradient concentrations of these antibiotics ranged from (0.001-1 mg/ml). 60 µl of each concentration of free form and hybrid nanociprofloxacin were put into well and incubated for 1 hour in the refrigerator, then incubated at 37 °C for 24 hours. The diameters of bacterial inhibition zone of each concentration for the free form and hybrid nanociprofloxacin were measured.

Statistical analysis

The Statistical Analysis System-SAS (2012) program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage and

least significant difference (LSD) test was used to significant compare between means in this study.

RESULTS

Conventional methods

The results of urine samples culture showed growth of creamy colonies of *E. coli* on nutrient agar plate, pink colonies on MacConcky agar plate, white and non-hemolytic colonies on blood base agar plate, and green with metallic shin colonies on eosin methylene blue (EMB) agar plate. The Gram staining of *E. coli* was showed a unicellular Gram-negative small bacilli organism. The results of API 20E test showed that the API 20E test was identified presence of *E. coli* in 27(36%) of 75(100%) patients clinically diagnosed with urinary tract infection.

Sensitivity test

The sensitivity test of *E. coli* isolates to certain concentrations of some antibiotics was referred to significant difference in the sensitive pattern of these isolates toward these antibiotics varied between sensitive (S) to intermediate (I), and resistance (R), as shown in (Table 1).

Analysis of extracted DNA of *E. coli* isolates

After performing of the DNA extraction from *E. coli* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1 % agaros gel at 7 volt/ cm for 1 hour (Figure 1).

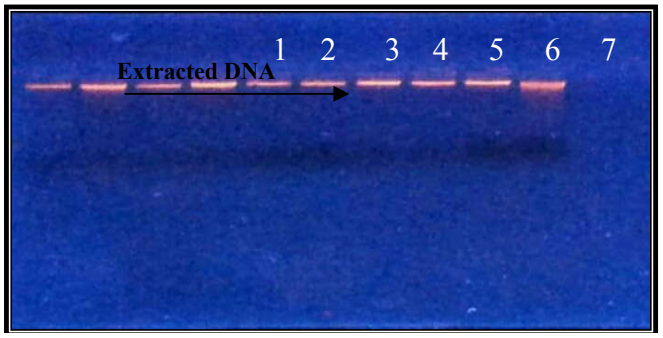

Figure 1. Gel electrophoresis of extracted DNA of *E. coli* isolates using 1% agarose gel at 7 volt/ cm for 1 hour. Lane 1-10: Extracted DNA

Table 1. Antibiotic susceptibility of *E. coli* isolates to certain antibiotics

No. of *E. coli isolate*	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Antibiotics	Antibiotic susceptibility													
AK	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AMC	R	R	R	R	R	R	R	I	R	R	R	R	R	I
GM	S	S	S	S	S	S	S	S	S	R	S	S	R	R
TOB	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SXT	S	R	S	R	S	I	R	S	S	R	R	S	R	R
NA	S	S	S	S	S	S	S	S	S	S	R	R	R	R
F	S	S	S	S	S	S	S	S	S	S	S	S	S	S
TET	S	R	S	I	S	R	S	S	S	R	I	S	R	S
TIC	S	R	R	R	R	R	R	S	R	R	R	R	R	R
AMP	S	R	R	R	R	R	I	S	R	R	R	R	R	R
MEM	S	S	S	S	S	S	S	S	S	S	S	S	S	S
LEV	S	S	S	S	S	S	S	S	S	S	S	S	R	S
CFM	S	R	R	S	R	S	S	S	S	S	S	R	R	R
NOR	S	S	S	S	S	S	S	S	S	S	S	S	R	S
NET	S	S	S	S	S	S	S	S	S	S	S	S	S	S
AZT	S	R	R	S	R	S	S	S	S	I	S	R	R	R
IPM	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CIP	S	S	S	S	S	S	S	S	S	S	S	S	I	S
PIP	S	R	R	R	R	R	R	S	R	R	R	R	R	R

No. of *E. coli isolate*	15	16	17	18	19	20	21	22	23	24	25	26	27
Antibiotics	Antibiotic susceptibility												
AK	S	S	S	S	S	S	S	S	S	S	S	S	S
AMC	R	R	R	R	R	R	I	R	R	R	R	R	R
GM	S	S	S	R	S	R	R	R	R	R	R	R	R
TOB	S	S	S	S	S	S	S	S	S	S	S	S	S
SXT	R	R	R	R	R	I	R	R	R	R	S	R	R
NA	R	R	R	R	R	R	R	R	R	R	R	R	R
F	S	S	S	S	S	S	S	S	S	S	S	S	S
TET	R	R	R	R	R	R	R	R	R	R	R	R	R
TIC	R	R	R	R	R	R	R	R	R	R	R	R	R
AMP	R	R	R	R	R	R	R	R	R	R	R	R	R
MEM	S	S	S	S	S	S	S	S	S	S	S	S	S
LEV	S	S	S	S	S	S	R	R	S	S	S	R	R
CFM	S	S	S	R	R	R	R	R	S	R	R	S	R
NOR	S	S	S	R	R	S	R	R	R	S	R	R	R
NET	S	S	S	S	R	I	R	R	S	S	S	R	R
AZT	S	S	S	R	R	R	R	R	R	S	R	S	S
IPM	S	S	S	S	S	S	S	S	S	S	S	S	S
CIP	S	S	S	R	R	R	R	R	R	R	R	R	R
PIP	I	R	R	R	R	R	R	R	R	R	R	R	R

S: Susceptible, I: Intermediate, R: Resistant

Analysis of PCR products of *E. coli PhoA* gene

The results of sigleplex PCR showed that all the 27 *E. coli* isolates of present study gave positive result for singlplex PCR of *pho*A gene represented by observation of the *pho*A gene product with ~903 bp molecular weight. The singleplex PCR products and 1 kb DNA ladder were resolved by electrophoresis. 5 µl of the PCR product were loaded on 1% agarose gel and run at 100 volt/ cm for 45 minutes. The gel was stained with ethidium bromide solution (0.5 µg/ ml) for 15-30 minutes; finally, bands were visualized on UV transiluminator at 350 wave length and then photographed by using photo documentation system. PCR result was considered positive for *E. coli* when there was presence of ~903 bp singleplex PCR product band on agarose gel electrophoresis, no amplification was observed with negative control (Figure 2).

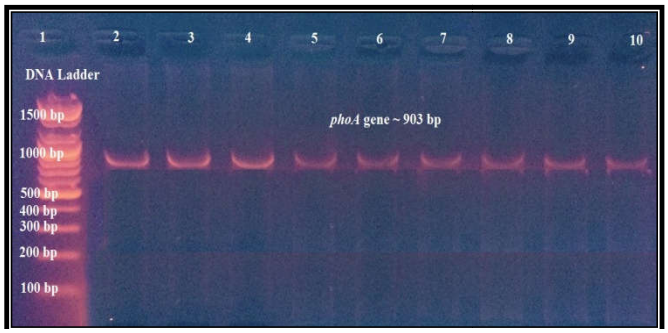

Figure 3. Gel electrophoresis of singleplex PCR products of *PhoA* gene for *E. coli* isolates using 1 % agarose gel at 7 volt/ cm for 1 hour. Lane 1: 1kb DNA ladder, lane 2-10: Singleplex PCR products of *PhoA* gene

Characterization of hybrid nanoantibiotics

Fourier transform infrared spectroscopy (FTIR)

(i)- FTIR of magnesium /aluminum -layered duple hydroxide carrier

The FTIR result of magnesium/aluminum-layered duple hydroxide (Mg/Al-LDH) carrier showed many of characteristic bands at specific frequencies as shown in (Figure 4), that include the band at 3527 cm^{-1} which was due to the stretching vibration of (O-H) bond and was a broad band because presence of interference from the different types of hydroxyl such as hydroxyl double layer and hydroxyl of physically adsorbed water, and the other characteristic band at 1383 cm^{-1} which was due to the nitrate group that existing between layers , whereas the last bands located between (400-600) cm^{-1} were due to the vibration of Al-O and Mg-O bonds.

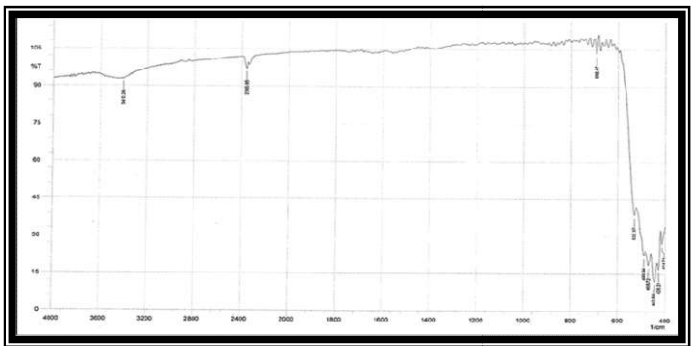

Figure 4. The FTIR of magnesium /aluminum- layered duple hydroxide (Mg/Al-DH) carrier

(ii)- FTIR of free ciprofloxacin

The FTIR result of free ciprofloxacin exhibited many of characteristic bands as shown in (Figure 5), that include the bands at 3535 cm^{-1} which were due to the stretching vibration of O-H bond of acid, the band at 3383 cm^{-1} which was due to the aminic N-H bond, the bands at 3091 and 3022 cm-1 which were due to the aromatic C-H bonds, the two bands at 2912 and 2841 cm^{-1} which were due to the asymmetric and symmetric stretching vibrations of the aliphatic C-H bonds respectively, the band at 1707 cm^{-1} which was due to the C=O bond of acid, whereas the band at 1622 cm^{-1} was due to the aromatic C-C bonds.

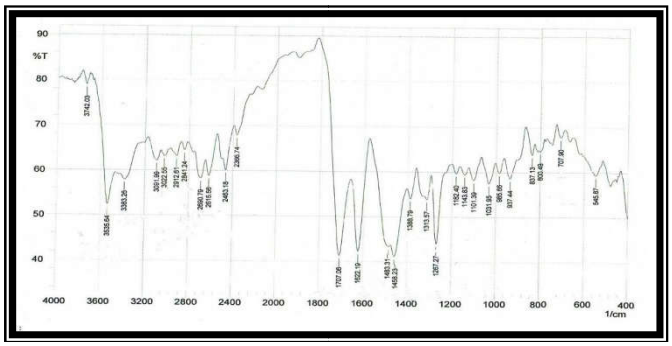

Figure 5. The FTIR of free ciprofloxacin antibiotic

(iii)- FTIR of hybrid nano-ciprofloxacin

The FTIR result of hybrid nanociprofloxacin (Mg/Al-CIP-LDH) showed appearance of some characteristic bands as shown in (Figure6), that include the band at 3425 cm^{-1} which was due to the NH_2 band that overlapped with the O-H bond, the bands at 2901 cm^{-1} and 2839 cm^{-1} which were due to the aliphatic C-H bonds, the band at 1621 cm^{-1} which was due to the C=O group, the bands at (1581, 1533 and 1485) cm^{-1} which were due to the aromatic C-C bonds, and finally, the bond at 1386 cm^{-1} was due to the stretching vibration of NO_3 group.

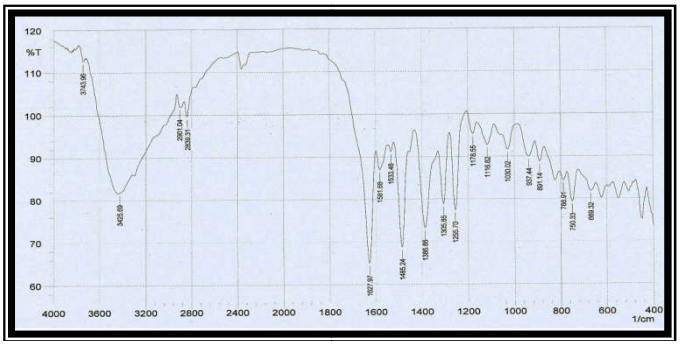

Figure 6. The FTIR of hybrid nano-ciprofloxacin (Mg/Al-CIP-LDH)

X-ray diffraction spectrum (XRD)

The X-ray diffraction spectrum of the hybrid nano-ciprofloxacin (Mg/Al-CIP-LDH) and hybrid nano-nalidixic acid (Mg/Al-NAL-LDH), and the (Mg/Al-LDH) carrier was performed to find out the difference in thickness of layer before and after the intercalation process of ciprofloxacin and Nalidixic acid between the layerd duple hydroxide. The result of X-ray diffraction spectrum of (Mg/Al-LDH) carrier showed three crystalline levels (003), (006) and (009) as shown in

(Figure 7). The first crystalline level 003 was appeared at the 10.47º angle with crystalline distance equal to 0.84 nm, the second crystalline level 006 was appeared at the 22.03º angle with crystalline distance equal to 0.41 nm, whereas the third crystalline level 009 was appeared at the 34.44º angle with crystalline distance equal to 0.26 nm.

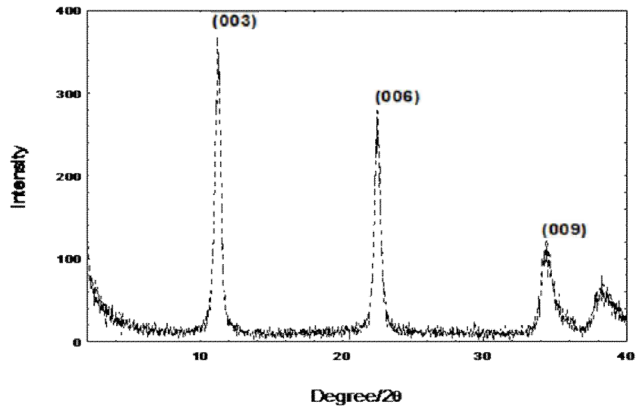

Figure 7. The X-ray diffraction spectrum of (Mg/Al-LDH) carrier

Also, the result of X-ray diffraction spectrum of the hybrid nano-ciprofloxacin (Mg/Al-CIP-LDH) in (Figure 8) referred to emergence of new bands are located at the angles (2 and 5) with crystalline distances (2.2 and 1.7) nm respectively, these indicating of insertion of ciprofloxacin antibiotic between the layers of LDH. these Both bands are indicative to crystalline levels (003) and (006), the crystalline level (009) seems to interfere with the level (003) for the layer duple hydroxide.

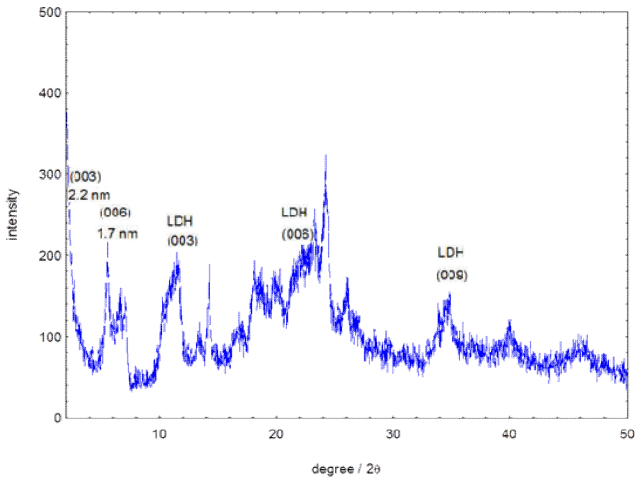

Figure 7. The X-ray diffraction spectrum of hybrid nano-ciprofloxacin (Mg/Al-CIP-LDH)

Atomic force microscope (AFM)

The result of atomic force microscope of hybrid nano ciprofloxacin (Mg/Al-CIP-LDH)showed two-dimensional image of hybrid nanociprofloxacin (Mg/Al-CIP-LDH) in molecules aggregation with observation of semi-spherical forms as shown in (Figure 8), whereas the (Figure 9) shows a three-dimensional image of surface section of the hybrid nanociprofloxacin with observation of molecular aggregation height that was up to 24.41 nm, which refers to preparation of the hybrid nano-ciprofloxacin from the free ciprofloxacin (CIP) and layers double hydroxide (LDH).

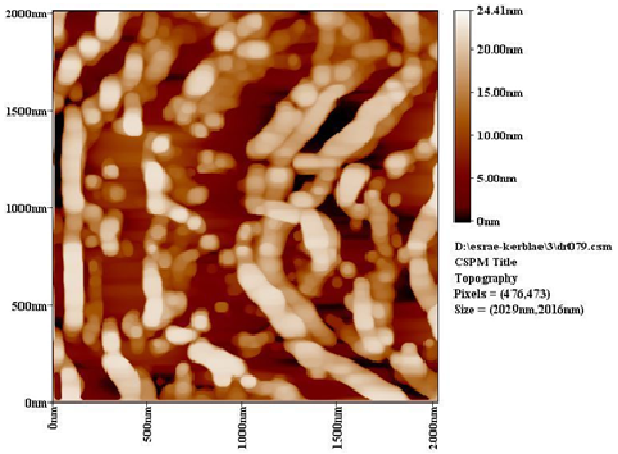

Figure 8. The two-dimensional image of hybrid nanociprofloxacin (Mg/Al-CIP-LDH) surface section by AFM

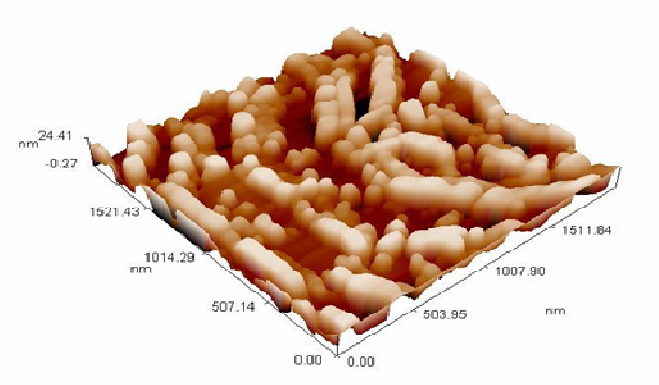

Figure 9. The three-dimensional image of the hybrid nanociprofloxacin (Mg/Al-CIP-LDH) surface section by AFM

Figure 10. The inhibitory activity of the free ciprofloxacin and hybrid nanociprofloxacin with concentration 1 mg/ml against the *E. coli* isolates

Also, the Table (2) showed that the average of particles size of the hybrid nanociprofloxacin (Mg/Al-CIP-LDH) was up to 98.00 nm and result of preparation this hybrid nanoantibiotic obtain particles with diameters 60, 70, 80, 90 and 100 nm and with rates 2.33, 6.98 , 9.30, 19.77 and 16.28% respectively.

Precise analysis of C, H and N elements

The results of precise analysis of C, H and N elements of the hybrid nanociprofolxacin (Mg/Al-CIP-LDH) exhibited that the percentages of carbon, hydrogen and nitrogen were 50.11, 5.83 and 8.35% respectively, whereas the results of precise analysis of C, H and N elements of the free ciprofloxacin antibiotic were 35.28, 5.63 and 5.76%, respectively.

Table 2. The average of particle sizes of the hybrid nanociprofloxacin (Mg/Al-CIP-LDH). (Average diameter:98.00 nm)

Diam.(nm)<	Vol.(%)	Cum.(%)	Diam.(nm)<	Vol.(%)	Cum.(%)	Diam.(nm)<	Vol.(%)	Cum.(%)
60.00	2.33	2.33	90.00	19.77	38.37	120.00	17.44	83.72
70.00	6.98	9.30	100.00	16.28	54.65	130.00	11.63	95.35
80.00	9.30	18.60	110.00	11.63	66.28	140.00	4.65	100.00

- Diam: Diameter, Vol.: Volum, Cum.: Cumulation

Table 3. The inhibitory activity of different concentrations of the free ciprofloxacin and hybrid nanociprofloxacin against the *E. coli* isolates

Concentration of antibiotic (mg/ml)		0.001	0.01	0.05	0.1	0.25	0.5	1	LSD value
E coli samples	Ciprofloxacin type	Inhibition zone of antibiotic (mm)							
IS1	CIP-FREE	0	25	31	34	35	36	38	6.41 *
	CIP-LDH	0	20	26	31	30	33	35	5.69 *
IS2	CIP-FREE	25	29	32	34	35	36	38	6.74 *
	CIP-LDH	20	25	30	31	32	33	35	6.02 *
IS3	CIP-FREE	27	31	34	35	36	37	39	7.63 *
	CIP-LDH	15	27	31	33	34	35	37	6.57 *
IS4	CIP-FREE	17	31	36	37	38	39	40	7.82 *
	CIP-LDH	16	28	33	34	35	36	38	6.55 *
IS5	CIP-FREE	23	31	34	35	36	38	40	7.09 *
	CIP-LDH	21	29	33	34	35	37	38	6.52 *
IS6	CIP-FREE	25	30	35	36	37	38	40	6.85 *
	CIP-LDH	21	29	33	34	35	37	38	5.99 *
IS7	CIP-FREE	25	31	34	35	36	38	39	6.02 *
	CIP-LDH	22	29	32	33	34	35	37	6.73 *
IS8	CIP-FREE	25	32	36	37	38	40	42	6.21 *
	CIP-LDH	20	29	34	36	37	38	40	7.09 *
IS9	CIP-FREE	24	31	35	36	37	40	42	8.13 *
	CIP-LDH	21	28	34	35	36	38	39	6.85 *
IS10	CIP-FREE	0	24	33	36	38	41	42	7.05 *
	CIP-LDH	0	21	30	34	37	39	40	6.98 *
LSD value		7.34 *	7.52 *	8.41 *	7.33 *	7.04 *	7.91 *	7.66 *	---

*** (P<0.05):** Significant difference

Detection of inhibitory activity of free and hybrid nano-ciprofloxacin against *E. coli* isolates

The results of detection of inhibitory activity for free ciprofloxacin and hybrid nano-ciprofloxacin against *E. coli* isolates exhibited that the free form of CIP and the hybrid form of CIP(Mg/Al-CIP-LDH) were showed the highest inhibition activity against the *E. co*li IS8 and *E. co*li IS10 isolates with inhibition zone diameter 42 and 40 mm respectively, by using both of these antibiotics with a concentration of 1 mg/ml and 0.5 mg/ml as shown in (Figure 10). Whereas the *E. co*li IS1 and *E. co*li IS2 isolates were less sensitive to these antibiotics represented by inhibition zone diameter 38 and 35 mm respectively. The Table (3) referred that the results of the statistical analysis explained presence of a significant increase $p<0.05$ in the inhibition zone average for each isolate of *E. coli* with increasing of antibiotics concentration that used in this study.

DISCUSSION

To sum up 27(36%) out of 75(100%) urine samples were detected for *E. coli* and 48(64%) out of 75 urine samples were detected for other bacterial species, the result of present study in agreement with result of study conducted by Sohail *et al.,* (2015) who studied urinary tract infection and isolated Gram negative and positive bacteria respectively, and they found that the most predominant isolated organism was Escherichia coli (62%) in culture positive urine samples, followed by *E. faecalis* (15%), Candida (14%), Pseudomonas (6%), *Klebsiella spp*. (1%), Proteus (1%), and Staphylococcus aureus (1%). In current study, the morphological characteristics of *E. coli* growth on nutrient agar were consistent with the findings of Al-Ferdous *et al.*, (2012) that refers to growth of circular, raised, smooth and creamy colonies of *E. coli* on the nutrient agar. In addition, the morphological characteristics of *E. coli* isolates of present study was similar to that characterized by Barcella *et al*., (2016) who showed growth of circular, pink color, lactose fermenting colonies of *E. coli* on MacConkey agar media and non-hemolytic colonies of these bacteria on blood base agar plate. Furthermore, the result of this study was matched with the result obtained by Islam *et al.*, (2014) which exhibited that the *E. coli* displayed green with metallic sheen colonies on eosin methylene blue (EMB) agar, also the result of Gram staining agrees with the findings of the study conducted by Zinnah *et al*. (2007) reported that *E. coli* is Gram-negative bacteria with rod shaped appearance that arranged in single or in pair and have growth of pink color colonies on MacConkey agar. Furthermore, the findings of *E. coli* isolates diagnosis by biochemical tests were consistent with the findings of Dash *et al*. (2012) when they used the biochemical tests for diagnosis of uropathogenic *E. coli* that isolated from urine sample of urinary tract infected patients. In the present study, the API 20E test was used for biochemical identification of *E. coli* isolates and this in agreement with study conducted by Yousef *et al*. (2013), referred to use of the API 20E test as a diagnostic biochemical tool for identification of *E. coli* isolated from different samples. Also, the results of antibiotic sensitivity demonstrated that 16(21.3%) of *E. coli* isolates out of 27(36%) of *E. coli* isolates were susceptible to cirprofloxacin (CIP) with a diameter of inhibition zone $\leq$ 15mm, and 10(13.3%) of *E. coli* isolates out of 27(36%) of *E. coli* isolates were susceptible to nalidixic acid (NAL) with a diameter of inhibition zone $\leq$ 13mm, these results agree with results of the study performed by Tansarlia *et al*. (2013) about antimicrobial susceptibility of *Enterobacteriaceae* causing

urinary tract infections, which found susceptibility of *E coli* against the ciprofloxacin and nalidixic acid varied from 68-91% and 52-92%, respectively and found ciprofloxacin had the highest activity among of oral fluoroquinolones antibiotics. Other study conducted by Sabir *et al*. (2014) also showed that quinolones are still active against urinary tract infections. The results of singleplex PCR showed that all the 27 *E. coli* isolates of present study gave positive result for singlplex PCR of *pho*A gene represented by observation of the *pho*A gene product with ~903 bp molecular weight. The result of this study go together with results of other studies referred to use of *phoA* gene as a target for detection of *E. coli* as this has been proven to be a universal marker (Kong *et al*., 1999; Thong *et al.*, 2011). The results of FTIR of magnesium /aluminum-layered duple hydroxide carrier approached with findings of different previous studies that they characterized the LDH carrier by using of FTIR method (Feng *et al*., 2006; Arizaga *et al*., 2009; Wang *et al*., 2010; Cheng *et al*., 2010; Parida *et al*., 2010). FTIR of free ciprofloxacin in the finding of present stud y convergent with finding of study carried out by Sahoo *et al*., (2011),who characterized the ciprofloxacin alone and ciprofloxacin/Carbopol composites by using the FTIR method. FTIR of hybrid nanociprofloxacin in the finding of this study agrees with finding of Patra *et al*. (2014) study who they used the hybrid nanociprofloxacin resulting from the ciprofloxacin-conjugated ZnO nanoparticles (ZN-CIP), and characterized that successful conjugation of ciprofloxacin by FTIR as confirmation method. The results of XRD for each LDH and hybrid nano-ciprofloxacin are correspond with the results of the study conducted by Khan *et al*., 2015 who they prepared nanohybrid of cefuroxime (CFO) with layered double hydroxide (LDH) and the intercalation process was confirmed by X-ray diffraction. The result of atomic force microscope of hybrid nano-ciprofloxacin (Mg/Al-CIP-LDH) agree with Prasannaraj *et al*. (2016) the synthesized AgNPs with average size 80-98 nm that exhibited a dose-dependent cytotoxicity against human liver and prostate cancer cell lines. The results of precise analysis of C, H and N elements of the hybrid nanociprofolxacin (Mg/Al-CIP-LDH) showed that the percentage of ciprofloxacin which loaded between the layers double hydroxide carrier could be estimated with 70.40% of this antibiotic.

The results of statistical analysis explained that there were significant differences in the inhibition average between the concentrations of free form of ciprofloxacine and hybrid nanociprofloxacin. Also, the (Table 3) referred that the results of the statistical analysis explained presence of a significant increase $p<0.05$ in the inhibition zone average for each isolate of *E. coli* with increasing of antibiotics concentration that used in this study. This results agree with Cunha *et al*., (2016) who used the disk diffusion method to find the effect of ciprofloxacin and silver nanoparticles (AgNPs) against *E coli* showed that increased activity of this nanoantibiotic against *E coli* and may represent a new therapeutic option for the treatment of bacterial infections.

Conclusion

The PhoA gene sequence, appeared to be useful genetic marker for determination of *E. coli* that isolated from urinary tract infection and could from other human infection. In addition, using of hybrid nano-antibiotics resulting from loaded of different antibiotics on LDHs carrier which is one of the inorganic delivery nanoparticles that possess some promising advantages such as low cost, good biocompatibility, low toxicity to mammalian cells, controlled release property, and full protection for loaded drugs, could be represented a promising future tool to treat and control urinary tract infection and other different bacterial infections.

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