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Antimicrobial susceptibility and Mollecullar detection of acc*(6´)-Ib* and *acc(6´)-II* genes among *Klebsiella pneumoniae* isolates collected from patients

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Abstract
Klebsiella pneumoniae bacteria are opportunistic pathogens , which causing community acquired and nosocomial infections. Fifty isolates of *K. pneumoniae* were obtained from 150 samples collected from different cases including urinary tract infections,bacteremia,burn,wound and pneumonia.The results of antimicrobial susceptibility exhibited that *K. pneumoniae* isolates were resistant to piperacillin (92%) ,ceftazidime and cefataxime(84%), azetreonam(78%) ,cefepime(74%), trimethoprime-sulfamethoxazole(64%), tobramycin(62%), ciprofloxacin(56%), gentamicin (50%) , Tetracycline(48%), amikacin (40%) , imepenem and ofloxacin (32%) and levofloxacin(28%). Mollecullar investigation of *acc(6´)-Ib* and *acc(6´)-II* genes revealed that *acc(6´)-Ib* gene was the most prevalent in which (68%) of isolates were harboured this gene . In contrast to *acc(6´)-II* gene was not detected in any one isolate. The gel electrophoresis revealed that the molecular weight of *acc(6´)-Ib* gene was 472bp. DNA sequence of *acc(6´)-Ib* gene showed the presence of a point mutation of subsituation. From the other side, the result of amino acids sequence revealed that was no effect of the *acc(6´)-Ib* gene mutation on the translation of the acc(6´)-Ib protein.

1.INTRODUCTION

Klebsiella pneumoniae , a member of the Enterobacteriaceae family, is a gram negative , rod shaped , non-motile , lactose fermentative , facultatively anaerobic with a mucoid capsule (Mohammed,2015). It is an opportunistic pathogen which infects immunocompromised patients or who are suffered from other infections, opportunistic *K. pneumoniae* can colonize human mucosal surfaces , this colonization can be progress to community acquired or nosocomial infections including urinary tract infections (UTIs),bacteremia,pneumonia,liver abscesses,cystitis and endogenous endophthalmitis (Martin *etal*.,2018).

K. pneumoniae represents one of the most important pathogens associated with antibiotics resistance especially multidrug resist (MDR) , *K. pneumoniae* employ several resistance mechanisms against different groups of antibiotics including production of antibiotic inactivating enzymes especially ESBLs , antibiotic target sites modification, membrane permeability change, alternation of efflux pump systems and metabolic pathway alternation(Kim *etal*.,2016). The overuse of antibiotics such as aminoglycosides making the resistance to this group of antibiotics widely spread and has influence it's therapeutic efficacy(Santajit and Indrawattana, 2016).

Aminoglycosides are bactericidal antibiotics that bind to 30S subunit of bacterial ribosomes resulting in inhibition of protein synthesis by supporting mistranslation and rejection of proofreading, aminoglycosides include many clinically important antibiotics such as gentamicin , amikacin , tobramycin and streptomycin , which are extremely used as an antimicrobial therapy in the treatment of nosocomial infections caused by *K. pneumoniae* (Hou *etal*.,2015). The principle adverse effects caused by these antibiotics are Nephrotoxicity , Ototoxicity and Neuromuscular toxicity . Aminoglycosides may also activate the production of strongly deleterious hydroxyl radicals (Chiem *etal*.,2017).

Resistance to aminoglycosides comes from several mechanisms including The production of aminoglycosides modifying enzymes (AMEs) such as acetyltransferases , phosphotransferases and adenyltransferases , target modification by mutation in proteins of ribosomes or in 16SrRNA or by methylation of 16SrRNA , alternation in membrane permeability and activation of efflux pumps (Hu *etal*.,2013).

The AMEs are the most common mechanisms to aminoglycosides resistance, aminoglycosides acetyltransferases (ACCs) are the major group of AMEs which can catalyze the transfer of actyl group from acetyl-coA to aminoglycoside molecules resulting in failure of their activity, the ACCs can be subdivided into four groups based on the position of acetylation occurrence in the aminoglycosides molecule , these groups including 1[ACC(1)] , 3[ACC(3)] , 2´[ACC(2´)] and 6´[ACC(6´)] , among ACCs groups the ACC(6´) represents the most common enzymes in gram negative bacteria especially *K. pneumoniae* , ACC(6´) enzymes can also be subdivided into two groups : ACC(6´)-I and ACC(6´)-II , ACC(6´)-I enzymes confer a resistance to several aminoglycosides especially amikacin, gentamicin C1a and C2 but not to gentamicin C1 , ACC(6´)-II enzymes provide a resistance to all forms of gentamicin but not of amikacin (Ramirez *etal*.,2013 ; Chiem *etal*.,2017 Fernandez-Martinez *etal*.,2017).

The genes encoding for these enzymes can be detected in most gram negative bacteria in integrons and transposons within plasmids and chromosomes (Chiem *etal*.,2017).

The aim of this study was to investigate for the resistance patterns against some antibiotics especially aminoglycosides and detect *acc(6´)-Ib* and *acc(6´)-II* genes encoding for aminoglycoside 6´-N-acetyl transferase type Ib and aminoglycoside 6´-N-acetyl transferase type II respectively in clinical samples of *K. pneumoniae* .

2. MATERIALS AND METHODS

2.1 Bacterial isolates collection

A total of 150 clinical samples were collected from patients who were suffered from different pathogenic cases including UTIs, bacteremia, burns and wounds infecions and pneumonia . The bacterial collection was performed during period from 10/10/2017 to 10/1/2018 from different hospitals in Baghdad,Iraq.

2.2 Bacterial Identification

All bacterial isolates were initially examined phenotypically by culturing on MacConkey agar,Blood agar and Eosin Methylene blue, then identified by using some biochemical tests including oxidase and catalase test (Salih *etal*., 2016). Finally identified by using Vitek2 system (BioMerieux, France) (Atef and Ghonaim,2014).

2.3 Antimicrobial susceptibility test

All identified *K. pneumoniae* isolates were tested for susceptibility to 14 antibiotics (Mastdiscs,UK) . These antibiotics including Cefepime(30 µg), Cefotaxime(30µg), Ceftazidime(30µg), Piperacillin(100µg), Azetreonam(30µg), Imipenem(10µg), Tetracycline(30µg), Trimethoprime-Sulfamethoxazole(25µg), Amikacin(30µg), Gentamicin(10µg), Tobramicin(10µg), Ciprofloxacin(5µg) , Levofloxacin(5µg), and Ofloxacin(5µg) .This test was performed according to Kirby-

Table 1 . The sequence of primers used in this study .

Genes		Primer Sequence (5´-3´)	Size (bp)	Refrence
aac(6′)-Ib	F	TTGCGATGCTCTATGAGTGGCTA	472	Hu *etal*.,2013
	R	CTGGAATGCCTGGCGTGTTT		
aac(6′)-II	F	CGACCATTTCATGTCC	542	Hu *etal*.,2013
	R	GAAGGCTTGTCGTGTTT		

Bauer disk diffusion using Muller-Hinton agar(Vandepitte *etal*.,2003). The results were interpreted depending on CLSI,2017.

2.4 DNA extraction

DNA was extracted from the bacterial isolates by using wizard genomic DNA purification kit (promega,USA) . The DNA concentration was measured by using Florometer Quantus (promega,USA).

2.5 genotyping detection of *acc(6´)-Ib* and *acc(6´)-II* genes

All extracted DNA of *K. pneumoniae* were screened for *acc(6´)-Ib* and *acc(6´)-II* genes using primers (Alpha DNA,USA) reported in table 1 . The amplification of DNA was performed by using Thermal Cycler(BioRad,USA), the Polymerase Chain Reaction (PCR) was carried out at a volum of 20µl , the reaction mixture was consisted of 10µl Go Taq Green Master Mix (Promega,USA) , 2µl template DNA, 1µl F-primer, 1µl R-primer and 6µl deionized sterile distilled water(Promega,USA), the PCR reaction of *acc(6´)-Ib* and *acc(6´)-II* genes was performed according to El-Badawy *etal*. (2017) in three steps: First initial denaturation at 95°C for 15min(1 cycle) and then denaturation of DNA template at 95°C for 1min to amplify DNA , annealing at 55°C for 1min, and finally extension at 72°C for 5min(30 cycles) and then final extension at 72°C for 3min (1 cycle) .

2.6 Separation of DNA bands

PCR products were run on 2% agarose gel with 5µl of ethidium bromide in 1X TAE buffer using DNA ladder (100-1500bp) supplied by (promega,USA) at 100 vol. for 80 min, the UV transilluminater was used for the observation of PCR products under 300nm UV light (Lee *etal*., 2012).

2.7 DNA sequence determination and analysis

The PCR product of the *aac(6′)-Ib* gene was transferred with 20µl of each F-primer and R-primer to Macrogen Inc. (South Korea) to make sequencing by genetic analyzer . The sequencing result was analyzed in comparison with the reference database available in the National Center for Biotechnology Information (NCBI) using BioEdit program version 7.1 (DNASTAR,Madison,WI,USA) (Hall *etal*., 2011). The variation was translated into amino acid sequences using expasy online program(Artimo *etal*., 2012).

3.RESULTS AND DISCUSSION

We obtained 50 isolates of *K. pneumoniae* from different clinical samples including urine(30%),blood(24%),burn(5%) and both wound and sputum (9%) (table 2) .Several studies revealed that the most common site of *K. pneumoniae* infections is UTIs followed by blood , sputum , burns , and wounds, Other studies collected *K. pneumoniae* from other cases including pus,stool,cerebrospinal fluid and catheters (Mohammed *etal*.,2015 ; Wasfi and Elkhatib,2016 ; Ali and Ismael *etal*., 2017 ; El-Badawy *etal*., 2017 ; Taitt *etal*., 2017).

Antimicrobial susceptibility test exhibited that most of *K. pneumoniae* isolates were resistant to piperacillin (92%) and the next largest proportions were resistant to ceftazidime and cefataxime (84%), azetreonam(78%), cefepime(74%), trimethoprime-sulfamethoxazole(64%). Tetracycline and imepenem showed the lowest resistance as (48%) and (32%) respectively . As regarding aminoglycosides we found that the resistant rates for tobramycin, gentamicin and amikacin reached (62%), (50%) and (40%) respectively. Concering quinolones, the resistant rates for ciprofloxacin,ofloxacin and levofloxacin reached (56%),(32%),(28%) respectively(table 3).

Table 2. The distribution of *Klebsiella pneumoniae* isolates

Samples	Isolates no.(%)
Urine	15(30)
Blood	12(24)
Sputum	9(18)
Wounds	9(18)
Burns	5(10)
Total	50(100)

Table 3. *Klebsiella pneumoniae* susceptibility pattern

Antibiotic	Sensitive no.(%)	Intermediate no. (%)	Resistant no. (%)
Cefepime	10(20)	3(6)	37(74)
Cefotaxime	8(16)	0	42(84)
Ceftazidime	8(16)	0	42(84)
Piperacillin	1(2)	3(6)	46(92)
Azetreonam	10(20)	1(2)	39(78)
Imipenem	26(52)	8(16)	16(32)
Tetracycline	26(52)	0	24(48)
Trimethoprime-Sulfamethoxazole	15(30)	3(6)	32(64)
Amikacin	23(46)	7(14)	20(40)
Gentamicin	24(48)	1(2)	25(50)
Tobramicin	17(34)	2(4)	32(62)
Ciprofloxacin	14(28)	8(16)	28(56)
Levofloxacin	33(66)	3(6)	14(28)
Ofloxacin	27(54)	7(14)	16(32)

The resistance to β-lactam antibiotics is due to the ability of *K. pneumoniae* isolates to produce extended spectrum beta lactamases (ESBLs) especially TEM and SHV which are acting actively gainat cephalosporins such as ceftazidime , cefotaxime and cefepime , the quite high frequencies of mutations in genes encoding for TEM and SHV enzymes leading to high diversity in these enzymes , therefore increasing the rates of antibiotics resistance, CTX-Ms have also been recognized in *K. pneumoniae* , which act against cefotaxime , Oxacillinases (OXAs) especially OXA-48 showed activity gainst imipenem , *Klebsiella pneumoniae* Carbapenemases (KPCs) also provide resistance against pipraicillin , ceftazidime , aztreonam and imipenem, AmpC β-lactamases confer resistance against aztreonam, all penicillins, and most cephalosporins ,the emergence of NDM-1 enzymes is responsible for the increasing of *K. pneumoniae* resistance rates to imipenem and that may represent a problem ,which threatens other antibiotics such as aminoglycosides and fluoroquinolones(Toroglu and Keskin,2011 ; Kim *etal*., 2016; Santajit and Indrawattana, 2016) . The loss of porin of outer membrane of *K. pneumoniae* such as OmpK35 and OmpK36 exhibit resistant to cephalosporins and carbapenems , the efflux pumps AcrAB-TolC in membrane of *K. pneumoniae* support the resistance against imipenem. (Almaghrabi *etal*., 2014 ; Santajit and Indrawattana, 2016).

The resistance ratios of cephalosporins ,which obtained by this study were close to the local study done by Ibrahim and Sharad (2013) , who referred that the *K. pneumoniae* isolates were resistance to ceftazidime (71%),cefotaxime(69%) and cefepime(69%) . While the Malaysian study performed by Mohsen *etal*.(2016) was incompatible with the results of this study when they found that their isolates were resistance to cefotaxime(35.5%) ,ceftazidime (34.5%) and cefepime(28.2%) .

The studies performed by Ibrahim and Sharad (2013) and Salih *etal*. (2016) from Iraq exhibited that (85%) and (77.35%) of isolates respectively were resistance to piperacillin , those results accepted with that of the current study , where as Varghese *etal*.(2016) from india reported that resistance rate reached 71.4% , so that this result didn't agree with the result of the present study.

By comparing the resistance result of imipenem with Shilpa K *etal*. (2016) from India and El-Badawy *etal*. (2017) from Egypt ,we observed that those studies were relatively similar with this study when their resistance percentages reached (43.33%) and (20.17%) respectively. In contrast ,Salih *etal*. (2016) and Ali *etal*. (2017) from Iraq found that the rate of resistant was (4.76%) and (93.86%) respectively .

Table 4. The distribution of *acc(6´)-Ib* in *Klebsiella pneumoniae* isolates

Samples	Isolates harbouring *acc(6´)-Ib* no.(%)	Isolates harbouring *acc(6´)-II* no.(%)
Urine	10(20)	0
Blood	12(24)	0
Burns	5(10)	0
Wounds	8(16)	0
Sputum	8(16)	0
Total	43(86)	0

The resistant result of aztreonam ,which recorded in the current study was concurred with those obtained by the local study of Ibrahim and Sharad (2013) and Jebur and Hammoudi (2014) , they reported that (69%) and (68.42%) of their isolates respectively were resistance to aztreonam , in contrast to Peerayeh *etal*. (2014) from Iran and Taitt *etal*. (2017) from Kenya recorded the low rates of resistance reached (39%) and (9%) respectively .

The resistance of tetracycline in *K. pneumoniae* is resulting from the alternations of the 30S and the 16S ribosomal subunits , cell permeability changes and expression of the efflux pumps AcrAB-TolC and OqxAB (Taitt *etal*., 2017). The resistance rate of tetracycline in this study was in agreement with that reported by Peerayeh *etal*. (2014) , Varghese *etal*.(2016) and Taitt *etal*. (2017) who found that (36.5%) , (45.7%) and (40%) of their isolates respectively were resistance to tetracycline , whereas Shahid *etal*. (2008) from India and Shabaa (2014) from Iraq mentioned that (11.47%) and (8.06%) of *K. pneumoniae* isolates respectively were resistance to tetracycline, it is clearly that those results were lower than that of the current study .

The resistance of *K. pneumoniae* to trimethoprime-sulfamethoxazole occurs because several mechanisms including changes of cell permeability, alternations of efflux pumps, genetic alternations in the target enzymes , horizontal gene transfer and expansion of clonal (Libecco and Powell, 2004).The studies of Makia *etal*.(2013) from Iraq , Shilpa K *etal*. (2016) , Varghese *etal*.(2016) and Taitt *etal*. (2017) exhibited that the resistance percentage of their isolates to the mentioned antibiotic was (56%) , (60%) , (50%) and (56%) respectively , those results were relatively close to the present study , but the results obtained by Rayes *etal*. (2011) from Iraq , Peerayeh *etal*. (2014) and Mohsen *etal*.(2016) didn't come in line with the result of this study when they recorded that the resistance percentages of their isolates were (83.3%) , (39%) and (38.2%) respectively.

K. pneumoniae acquire the resistance to aminoglycosides through aminoglycosides modification enzymes , 16srRNA methylase and alternation in the efflux pumps AcrAB-TolC and KpnEF (Peerayeh *etal*. 2014; Hu *etal*.,2013). The research of Shahid *etal*. (2008) showed that (52.45%),(42.62%) and (36.06%) of isolates were resistance to tobramicine, amikacine and gentamicine respectively. Also Shilpa K *etal*. (2016) exhibited that (58.06%) , (37.50%) and (33.33%) of isolates were resistance to tobramicine, , amikacine and gentamicine respectively , these rusults were relatively similar to that of this study . While the resistance rates obtained by lotfollahi *etal*. (2015) from Iran were in different with this study when they found that (26.9%) , (21.2%) and (6.1%) and of isolates were resistance to gentamicine , tobramicine and amikacine respectively .

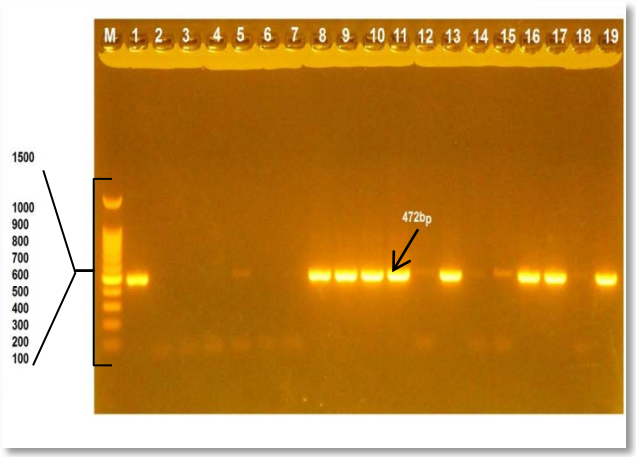

Figure 1 : Electrophoresis of PCR products of acc(6´)-Ib gene on 2% agarose at 100vol./80min. Line M: DNA ladder (100-1500bp). Lines 1,5,8,9,10,11,12,13,14.15,16,17,18,19 *Klebsiella pneumoniae* PCR positive isolates . Lines 2,3,4,6,7 *Klebsiella pneumoniae* PCR negative isolates.

K. pneumoniae combine several mechanisms for quinolones resistance including mutations in target site gene , production of MDR efflux pumps , modification of enzymes and targeting the protection proteins (Santajit and Indrawattana, 2016).In the study of El-Badawy *etal*. (2017) the ratio of resistance reached (44.73%) and (38.59%) for ciprofloxacin and levofloxacin repectively . For ofloxacin, toroglu and keskin (2011) from Turkey found that (27%) of isolates were resistance to this antibiotic . These results concurred with the results of this study . In contrast Seyedpour and Eftekhar (2014) from Iran showed resistance rates were lower than of the current study when they found isolates were resistance to levofloxacin (13.5%), ciprofloxacin (7.7%) and ofloxacin (7.7%) respectively.

The fluoroquinolones and imipenem represent an effective antimicrobial therapies against *K. pneumoniae* , but the extensive use of imipenem leading to highly emergence of KPCs ,and consequently poses a risk problem in the control of healthcare-associated infections (Santajit and Indrawattana, 2016). Furthermore, the transmission of fluoroquinolones and imipenem resistance genes by conjugation among *K. pneumoniae* strains has increased the problem of MDR *K. pneumoniae* (Hou *etal*.,2015) .

The combination of antibiotics therapies has currently been used to limited the evolution of multidrug resistant *K. pneumoniae* and the antibiotics level used in the treatment of the *K. pneumoniae* infections (Kim *etal*., 2016).

Genotyping detection for *acc(6´)-Ib* and *acc(6´)-II* genes revealed that the *acc(6´)-Ib* gene was present in 43(86%) isolates ,in Almaghrabi *etal*.(2014) research , 98% of *K. pneumoniae* isolates were harboured *acc(6´)-Ib*, El-Badawy *etal*. (2017) detected

acc(6´)-Ib in 88% of isolates .Those results accepted with that of this study . In contrast to Peerayeh *etal*. (2014)and Fernandez-Martinez *etal*. (2017) who reported that (42.6%) and (44.6%) of their isolates respectively were harboured *acc(6´)-Ib* and these results incompatible with that of this study .In other hand *acc(6´)-II* was not detected in any one isolate , this result agreement with the study of El-Badawy *etal*. (2017) . this means the acc(6´)-Ib enzyme was the most prevalent type of AMEs among *K. pneumoniae* isolates .

The gel electrophoresis revealed that the molecular weight of *acc(6´)-Ib* gene was 472bp. The acc(6´)-Ib is the most extensively distributed enzyme among producing gram-negative bacteria and provides resistance to clinically relevant aminoglycosides (Almaghrabi *etal*. 2014 ; Hu *etal*.,2013).

Although some *K. pneumoniae* isolates of this study were harbouring *acc(6´)-Ib* gene but don't appear resistance to aminoglycosides used in this study , this difference between phenotype and genotype of aminoglycosides resistance may be due to the mutations occurrence in the gene which in turn prevented the emergence of AMEs as an important mechanism of aminoglycosides resistance(Hou *etal*.,2015).

The analyzed sequence result for the KP23 isolate exhibited that there is one mutation in the DNA of the *acc(6´)-Ib* gene, this mutation was point mutation of substitution,this substitution was transition.Transition replacement of pyrimidin base with another pyrimidin (C>T) at the position 143492 . The result of protein translation revealed that a silent mutation in the gene , in which didn't affect in the amino acids sequence of the protein .The DNA sequence of *Klebsiella pneumoniae aac(6´)-Ib* gene ,which analyzed in this study was recorded in the NCBI website at the link: https://www.ncbi.nlm.nih.gov/nuccore/LC373256

Referring DNA sequences

TTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAG
TGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTAC
AGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCAC
TCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTATGCCC
AGTCGTACGTTGCTCTTGGAAGCGGGGACGGACGGTGGGAAGA
AGAAACCGATCCAGGAGTACGCGGAATAGACCAGTTACTGGCG
AATGCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTC
GAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAG
ATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGAT
GCTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCAC
CCCATATGGTCCAGCCGTGTACATGGTTCAAACACGCCAGGCAT
TCGAG

Mutant DNA sequences

TTGCGATGCTCTATGAGTGGCTAAATCGATCTCATATCGTCGAG
TGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTAC
AGGAACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCAC
TCCATACATTGCAATGCTGAATGGAGAGCCGATTGGGTATGCCC
AGTCGTACGTTGCTTTTGGAAGCGGGGACGGACGGTGGGAAGA
AGAAACCGATCCAGGAGTACGCGGAATAGACCAGTTACTGGCG
AATGCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTTC
GAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAG
ATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCGAT
GCTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCAC
CCCATATGGTCCAGCCGTGTACATGGTTCAAACACGCCAGGCAT
TCGAG

Figure 2 : DNA sequence of *Klebsiella pneumoniae aac(6´)-Ib* gene comparing with NR5632 strain chromosome , complete genome , sequence ID:gb CP025143.1

Referring protein sequences

MTNSNDSVTLRLMTEHDLAMLYEWLNRSHIVEWWGGEEARPTL
ADVQEQYLPSVLAQESVTPYIAMLNGEPIGYAQSYVALGSGDGRW
EEETDPGVRGIDQLLANASQLGKGLGTKLVRALVELLFNDPEVTKI
QTDPSPSNLRAIRCYEKAGFERQGTVTTPYGPAVYMVQTRQAFER
TRSDA

Figure 3 : The sequence of amino acids of the protein aac(6´)-Ib resulting from the translation of the *aac(6´)-Ib* gene .

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