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RESEARCH ARTICLE

Studying the Pattern of Phenotypic Resistance to some Antibiotics for Local Isolates of Enterobacter Cloacae and its Evolutionary Relationship with Global Isolates

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Abstract

26 isolates of Enterobacter cloacae from 264 specimens from different clinical sources and from inpatients for ages ranged from one Week to 73 years from 15/12/2016 to 18/7/2017collected from two hospitols of AL-Diwaniyah city. The isolation and diagnosis process was based on the results of phenotypic and biochemical tests and the Vitek system .The sensitivity of the isolates for antibiotics were tested against 15 antibiotic using disk diffusion method , The results showed an absolute resistance (100%) for Amoxicillin, Cephalothine and Doxycycline, while their sensitivity to Amikacin , Gentamicin, Tetracycline, Nitrofurantoin and Nalidixic acid were various, they resisted it at percentage of 11.53 %, 34.61% , 73.07% , 34.61% and 23.07% respectively . For the rest of the Cephalosporin group (Cefoxitin, Cefotaxim and Ceftriaxone) , the isolates were resisted them with percentage 65.38%, 57.69% and 34.61%, respectively , While all isolates (100%) were sensitive to Chloramphenicol, Ciprofloxacin, Impenem and Meropenem . Twenty-four isolates (92.30%) were able to produce ß-lactamase. PCR was used to amplify HSP60 gene which used to determine the sequence of nucleotides and draw the phylogenic tree and find the relationship between the current isolates of this study with global isolates.

Keywords: *Enterobacter cloacae, HSP60 gene, PCR, evolutionary relationship.*

Introduction

Enterobacter cloacae are saprophytic microorganisms, live in digestive system as normal flora and can be isolated from patients suffering from diarrhea, urinary tract infection and bloodstream infection [1]. It gram negative bacteria, bacilli, motile, facultative anaerobic and opportunistic can transform to pathogens [2].

E. cloacae is able to resist multi drugs which are coded with chromosome as resistance of penicillin and the first three generation of cephalosporin or extra genetic elements as plasmids[3,4]. Many of these bacteria are able to produce extended spectrum ß-lactamases enzymes which hydrolysis penicillin. monobactam, carbapenems and cephalosporin and coded with plasmids mostly [5].

*Enterobacter*sp. Have gene called *hsp60* which used as tool for molecular identification of this genus and make DNA sequencing and study the evolutionary relationship and compare it with global isolates [6].Because of pathogenic of *E. cloacae* and reducing local studied about it, this study aims to appraising the phenotype resistance of this bacteria and it's capable to produce ß-lactamases enzymes besides draw phylogenic tree of it.

Materials and Methods

Samples Collection

264 sample were collected from different clinical sources and from inpatients in AL-Diwaniyah women's and children's educational and AL-Diwaniyah General Educational hospitals for ages ranged from one Week to 73 years for the period from 15/12/2016 to 18/7/2017 ,include 90 samples of stool from patients with diarrhea , 135 samples of urine (58 samples took from urine catheter sacs and 77 samples took directly)

besides 38 swaps from urine catheter tube and one sample from blood of patient suffering from septicemia.

The Isolation and Identification

All samples cultured on routine media like Macconkey agar and Blood Agar (Difco, USA) and incubated at 37c for 20-24 h. the results of culture used to identify *E.cloacae* besides biochemical tests, gram stain. Vitek 2 Compact system (Biomerieux , USA) was used for final identification .

Susceptible of Antibiotic Test

Disc Diffusion Method was used to make the test depend on Kirby and Bauer (1966)by culture *E. cloacae* on blood agar , and select pure bacterial colony to culture it on Muller Hinton agar (Biolife, Italy)with swaps then put the discs of antibiotic including Amoxicillin, Cephalothine, Doxycycline, Amikacin , Gentamicin, Tetracycline, Nitrofurantoin, Nalidixic acid, Cefoxitin, Cefotaxim , Ceftriaxone , Chloramphenicol, Ciprofloxacin, Impenem and Meropenem (6 discs of antibiotic for each petri dish), the dishes incubated for 16-18 h at 35±2 °C , then reading of the results depend on inhibition diameters and compare it with CLSI [7] .

Production of β- lactamase Enzymes Test

Strips test of ß- lactamase Enzymes were used for quick detection about the ability of *E. cloacae* to produce these enzymes which are responsible on beta lactam drugs resistant in gram negative besides the positive. One pure well grown colony was transferred with sterile loop and streaked on the moisture test strip, if the color of the strip changes into reddish pink during five minute, that's mean it's positive interaction.

Polymerase Chain Reaction Assay (PCR)

DNA Extraction

Presto Mini gdna Bacteria Kit (Geneaid , South Korea) used to extract the nuclear acid DNA depend on the steps of the procedure which are found with the kit .DNA is kept in refrigerator at 2-8 °C to use in genetic assay .

Amplifying of Heat Shock Protein gene (*HSP60*):

HSP60 amplified by using polymerase chain reaction mixture which contains from: Prime Taq Premix (GeNet Bio, South Korea), primers of *HSP60* (Table 1), DNA template and free nuclease water by using thermal cycles program (Table 2).

Table 1: Primer used in this study

gene	PCR primer	Primer sequence 5' _3'	Tm °C	PCR product (nt)	Reference
HSP60	Hsp60-F	GGT AGA AGA AGG CGT GGT TGC	61.8	341	8
	Hsp60-F	ATG CAT TCG GTG GTG ATC ATC AG	60.6		

Table 2: Thermal cycle's program of Hsp60 gene primer

Initial denaturation		94˚C for 5 min.
Cycle conditions	**denaturation**	94˚C for 30 sec.
	annealing	57.5˚C for 30 sec.
	extension	72˚C for 60 sec.
Final Extension		72˚C for 5 min.
Cycles No.		30

Agarose Gel Electrophoresis

Method of Sambrook [9] used to Electrophoresis PCR product of *HSP60* gene by using Agarose salts , Tris Borate EDTA (TBE), ethidium bromide and Loading Dye at 80V/1 H , then UV ray used to observe the bands and compare it with ladder .

DNA Sequencing and Evolutionary Relationship

21 samples of PCR produces of *HSP60* gene of *E. cloacae* sent to Macrogene Co. (South Korea) to make DNA sequencing for these bacteria, nucleotides exhibited on gene bank for getting accession number for each isolate, then drew phylogenic tree by using NCBI-Blast Alignment.

Results and Discussion

Isolation and Identification

26 isolates of *E. cloacae* from 264 samples from different sources including urine, stool, catheter system of urine and blood were got in this study depending on their cultural

characters, microscopic, biochemical and finally using of Vitek 2 Compact system which can diagnose this bacteria in high accuracy reach to 100% nearly and it could divide our bacteria into two Subspecies (Table 3). From result we found that the rate of our isolation of *E. cloacae* was 9.84% including 5.3% from urine, 1.8% from stool for patients with diarrhea, 1.13% for each of catheter sacs and swaps of catheter tube and 0.37% from blood of patient suffering from bacteremia.

The total rate of isolation was similar to 10 who get 9.61% of this bacteria .The catheter systems are gates to entrance *Enterobacter i*nto blood stream[11], and the role of *Enterobacter* as causing of diarrhea is common because these bacteria are opportunistic and can transfer from normal flora to be pathogenic and striking the human body and its tissues especially when make surgical operations or put the urine catheter systems for a long time which can contribute in spreading of bacteria and causing Nosocomial infections[12].The ability of *E. cloacae* for coexisting out of intestine was recorded from [13] who consider that *Enterobacter* spp.

Is one of ten common bacterial genuses causing bacteremia in hospitals especially for immunocompromised patients besides the National Nosocomial Infections Surveil-lance System (NNIS) consider that *Enterobacter* sp. responsible on 5-7% of human infection which was acquired from hospitals in USA from 1976 -1989.

Table 3: subs pies of Isolates in this study and its source

No. of isolate	Source of isolate	Name of isolate
1	Urine	*E. cloacae* subspecies *cloacae*
2	Catheter tube	*E. cloacae* subspecies *cloacae*
3	Stool	*E. cloacae* subspecies *cloacae*
4	Urine	*E. cloacae* subspecies *dissolvens*
5	Urine	*E. cloacae* subspecies *cloacae*
6	Catheter sac	*E. cloacae* subspecies *cloacae*
7	Blood	*E. cloacae* subspecies *dissolvens*
8	Urine	*E. cloacae subspecies cloacae*
9	Urine	*E. cloacae subspecies cloacae*
10	Urine	*E. cloacae subspecies cloacae*
11	Stool	*E. cloacae subspecies cloacae*
12	Catheter tube	*E. cloacae* subspecies *dissolvens*
13	Urine	*E. cloacae* subspecies *cloacae*
14	Urine	*E. cloacae* subspecies *cloacae*
15	Stool	*E. cloacae* subspecies *cloacae*
16	Catheter sac	*E. cloacae* subspecies *dissolvens*
17	Urine	*E. cloacae* subspecies *cloacae*
18	Urine	*E. cloacae* subspecies *cloacae*
19	Stool	*E. cloacae* subspecies *dissolvens*
20	Urine	*E. cloacae* subspecies *dissolvens*
21	Catheter tube	*E. cloacae* subspecies *cloacae*
22	Stool	*E. cloacae* subspecies *cloacae*
23	Urine	*E. cloacae* subspecies *cloacae*
24	Catheter sac	*E. cloacae* subspecies *dissolvens*
25	Urine	*E. cloacae* subspecies *dissolvens*
26	Urine	*E. cloacae* subspecies *cloacae*

E. cloacae Susceptible of Antibiotic

The results of Susceptible of Antibiotic of *E. cloacae* (Figure 1) showed that all isolates (100%) were resisted to amoxcillin, cephalothin and doxcyclin while their sensitivity to Amikacin , Gentamicin, Tetracycline, Nitrofurantoin and Nalidixic acid were various, they were resisted it at percentage 11.53 % , 34.61% , 73.07 % , 34.61 % and 23.07 % respectively .

For the rest of the Cephalosporin group (Cefoxitin, Cefotaxim and Ceftriaxone), the isolates were resisted them with percentage of 65.38%, 57.69% and 34.61%, respectively. All isolates (100%) were sensitive to Chloramphenicol, Ciprofloxacin, Impenem and Meropenem which mean their successful drugs to treat the infections caused by E. *cloacae.*

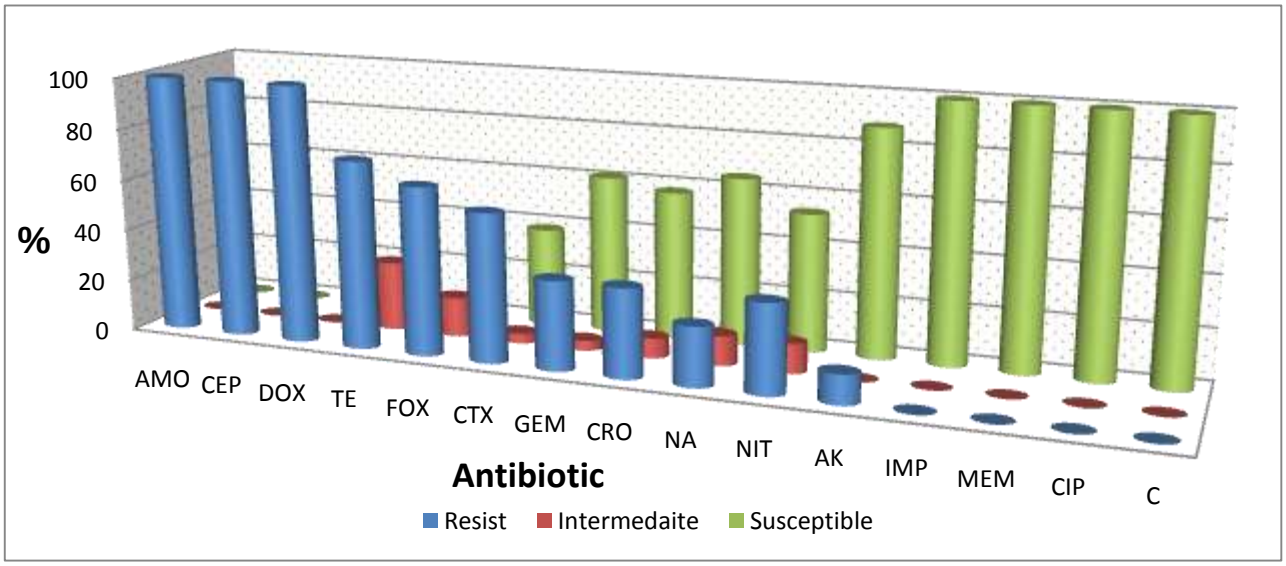

Figure 1: Susceptible of Antibiotic in *E. cloacae* isolates
AMO=Amoxcillin , CEP=Cephalothin, Dox= Doxcyclin , TET= Tetracycline , FOX=Cefoxtin , CTX= Cefotaxime , GEM=Gentamicin , CRO= Ceftriaxone , NA= Nalidixic acid , NIT=Nitrofurantoin , AK=Amikacin , IMP= Imipenem ,MEM=Meropenem , CIP= Ciprofloxacin , C= Chloramphenicol

The absolute resistance to Amoxcillin in *E. cloacae* record from number of researcher as [14,15] and it is due to the ability of these bacteria to produce ß-lactamases enzymes which coded by chromosome besides these enzymes are common in species of *Enterobacter* genus so that give it selective character under treatment pressure [2]. ß-lactamase class A can resolve beta lactam ring of penicillin and make it inactive toward bacteria , while high production of ß-lactamase class C can resolve penicillin and cephalosporin [2,16].

The partial resistance of *Enterobacter* to the third generation of cephalosporin like Cefotaxime and Ceftriaxone due to chromosomal Amp C cephalosporinase enzymes [16], or happening mutation effect on ß-lactamase class A include CTX-M which has extended spectrum in cephalosporin lysis [17], or because of ability of *E. cloacae* to produce carbapenemases class A enzymes(it has 2 group of these enzymes IMI-2 and NMC-A) which are common in enterobacteraceae and it can analyze most of beta lactam antibiotic like penicillin , cephalosporin , carbapenem and aztreonam [18].

Resistance of our isolate to aminoglycoside especially gentamicin due to modify enzymes for aminoglycoside like acetyltransferases and adenyltransferases besides of the exchange of plasmids and genetic transfer elements increase of bacterial resistance to these antibiotic [2].

The bacterial resistance to tetracyclins in our isolates are clear (absolute resistance to doxcyclin and 73.07% to tetracycline and may be due to ribosomal protection or by using efflux pumps coded by genes carried on chromosome or plasmids especially in gram negative to export tetracyclins out of bacterial cell and protect it from bacteriostatic effects of tetracyclins, besides of the ability of mutation existing in targets of these antibiotic19,20. Most of the isolates of *E. cloacae* were sensitive to quinolones (Ciprofloxacin and Nalidixic acid) which were used in this study.

The active effect of these antibiotics especially Ciprofloxacin was recorded from [21, 22] who got sensitivity rate (100, 96) % respectively. Mechanism of quinolones' effect is inhibit duplication and transcriptions of DNA by binding with DNA gyrase the essential aim for quinolones in gram negative bacteria23,while 24 showed that the increasing resistance to quinolones as Nalidixic acid is due to chromosomal mutations or caused by plasmids carried genes called *QnrA* gene although anther studies showed that *QnrA* geneis unable to resist ciprofloxacin we found it alone and this may explain the reason of the sensitivity of all our isolates to Ciprofloxacin.

Carbapenems like Imipenem and Meropenem besides Chloramphenicol as antibiotic from phenicol group proved their activity to kill all *E. cloacae* isolates which did not show any

resistance towards these bacteria; therefore it is a successful antibiotic to the treatment of infection with *E. cloacae* , and this result agree with [25].

Chloramphenicol is considered from extend spectrum and bacteriostatic antibiotic which can inhibit protein synthesis in bacteria by binding with ribosomal subunit 50 S and interaction with peptidyl transferase and blocking the elongation of peptide chain [23].

Because of high toxicity of chloramphenicol, recede use of this antibiotic for human in forth-going nations and rarely use for systematic infections but can use locally26and there is a warning when using in pregnancy and people who have allergy to it, besides chloramphenicol can cause anemia because of the killing effect on normal flora which provide the human body with amount of vitamin K [27].

Among all our isolates of *E. cloacae* we found just 9 isolates (34.61%) resistant to nitrofurantoin. Resistance of *E. cloacae* to nitrofurantoin depends on using of efflux pumps to get out this antibiotic from bacterial cell [28]. Nitrofurantoin can use to treat many infection caused by gram negative especially intestinal infection and urinary tract infections (UTI) because of its effect on bacterial DNA , and it is considered as safely drug in many countries including USA to treat UTI in pregnant women depending on laboratories results and experiments on animals upon injected with nitrofurantoin [29].

B-lactamase Production

Strips saturated with chromogenic cephalosporins used to test the ability of *E. cloacae* isolates to produce ß-lactamase enzymes. Positive results depend on the changes of strip color to reddish pink (Figure 1) as a result of refraction amide bond of beta lactam ring by ß-lactamase and changes color of nitrocefin due to high sensitivity of this material to ß-lactamase which are common in gram negative and positive .

Our results for this test showed that 24 isolates (92.30 %) were produced ß-lactamase while two isolates (one from female has diarrhea and anther from urine catheter sac for inpatient at fractions unit) were unable to produce these enzymes.

The production of ß-lactamase test which is called sometimes (nitrocefin test) has high accuracy, speed and sensitivity for ß-lactamase or hydrolysis of beta lactam ring and this sensitivity are due to easily dissolving of nitrocefin in these enzymes and iodine or pH indicator responsible on color change of ß-lactamase strips30.

Figure 1: Strips test of β- lactamase: (upper strip= (-) result, bottom strip = (+) result

Amplification of HSP60 Gene

Monoplex PCR were used to amplify hsp60 gene which can use to identify Enterobacter genus and to find DNA sequencing to study evolutionary relationship among our positive isolates with global isolates of E. cloacae by using NCBI-Gene Bank Global .PCR technique showed all our isolates were followed Enterobacter spp. (Figure 3 , A and B). HSP60 gene has more accuracy than 16SrRNA gene because the ability of 16SrRNA for recognize among close species of enterobacteraceae especially Enterobacter spp. is weak compared with hsp 60 gene [31].

Finding Evolutionary Relationship of Locally Isolates of Enterobacter spp

We get nucleotides sequencing for 21 isolates of E. cloacae which sent to Macrogene company in South Korea , then offered in NCBI-Gene Bank database for getting accession number for each isolate (table 4).Phylogenic tree were drawn by using MEGA6 program and analysis of HSP60 sequences of E. cloacae (Figure 3). The similarity rate of our isolates comparing with global isolates were in range between 99-100 % , and most of our isolates were similar to USA and South Korea isolates besides number of our isolates were similar with another global isolates from Tanzania , Switzerland and China.

Table 4: Nucleotides Sequences and Accession No. of our isolates

No. of Sequence	source	Accession No.
1	Urine	MH119313
2	Catheter tube	MH119314
3	Stool	MH119315
4	Urine	MH119316
5	Urine	MH119317
6	Catheter sac	MH119318
7	Urine	MH119319
8	Urine	MH119320
9	Urine	MH119321
10	Stool	MH119322
11	Urine	MH119323
12	Stool	MH119324
13	Catheter sac	MH119325
14	Urine	MH119326
15	Urine	MH119327
16	Stool	MH119328
17	Urine	MH119329
18	Urine	MH119330
19	Catheter sac	MH119331
20	Urine	MH119332
21	Urine	MH119333

The differences among the isolates of same species may be due to the structural differences among these isolates. In study of Population Genetics of the Nomenspecies *E. cloacae* achieved by [8] showed differences among bacterial groups of *E. cloacae* due to differences of specific amino acids which found in heat shock protein coded by *HSP60* e.g. *E. cloacae* subspecies *cloacae* have specific proteins in the position 430 and 466 differ from proteins which are found in the position 430 of *E. cloacae* subspecies *dissolvens*.

Using of HSP60 gene which is called *groEL* or *cpn60* is successful and a useful application for classification of bacteria besides of its ability to analyze the evolutionary relationships and draw phylogenic tree [32].

Conclusion

E. cloacae are multi drugs resistance as amoxillin , cephalothin and Doxcyclin and the best drugs for treatment infections caused by this bacteria are chloramphenicol , ciprofloxacin ,impenim and meropenem besides this study proved that most of *E. cloacae* able to produce ß-lactamases enzymes which increase its virulence. We find high identity among our isolates and the global isolate by using nucleotides sequences and their phylogenic tree.

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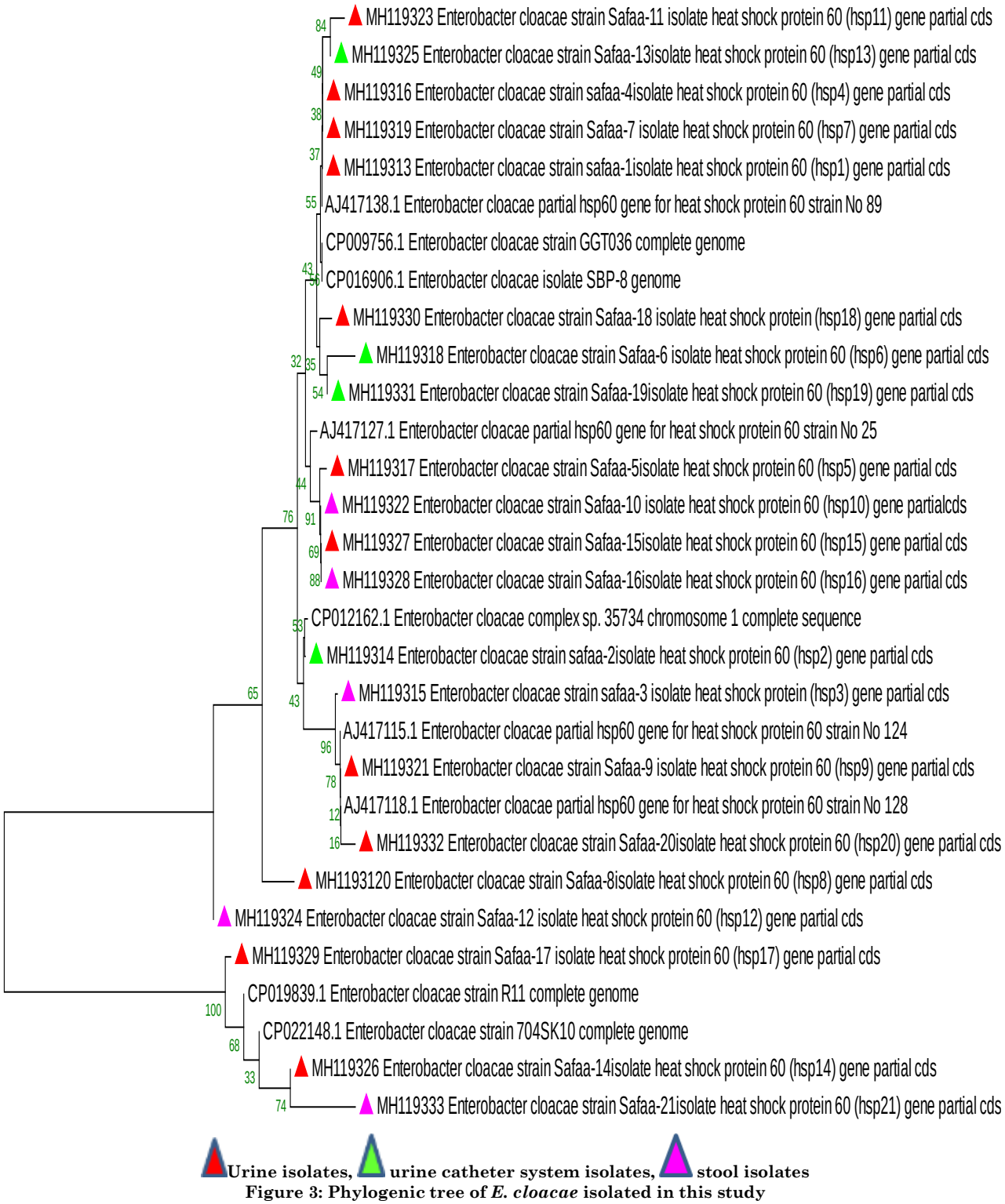

Figure 3: Phylogenic tree of *E. cloacae* isolated in this study

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