Examination of uropathogenic *Escherichia coli* strains conferring large plasmids

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ABSTRACT

Suhartono (2010) Examination of uropathogenic Escherichia coli *strains conferring large plasmids. Biodiversitas 11: 59-64.* Of major uropathogens, *Escherichia coli* has been widely known as a main pathogen of UTIs globally and has considerable medical and financial consequences. A strain of UPEC, namely *E. coli* ST131, confers a large plasmid encoding cephalosporinases (class C -lactamase) or AmpC that may be disseminated through horizontal transfer among bacterial populations. Therefore, it is worth examining such large plasmids by isolating, purifying, and digesting the plasmid with restriction enzymes. The examination of the large plasmids was conducted by isolating plasmid DNA visualized by agarose gel electrophoresis as well as by PFGE. The relationship of plasmids among isolates was carried out by *Hpa*I restriction enzyme digestion. Of 36 isolates of *E. coli* ST 131, eight isolates possessed large plasmids, namely isolates 3, 9, 10, 12, 17, 18, 26 and 30 with the largest molecular size confirmed by agarose gel electrophoresis and PFGE was ~42kb and ~118kb respectively. Restriction enzyme analysis revealed that isolates 9, 10, 12, 17 and 18 have the common restriction patterns and those isolates might be closely related.

Key words: large plasmids, uropathogenic *E. coli*, electrophoresis, RE, PFGE.

INTRODUCTION

Urinary tract infections (UTI), the most common infectious diseases triggering significant medical and financial implications, are defined as the presence of microbial pathogens within urinary tracts identified by a wide spectrum of symptoms ranging from mild irritative voiding to bacterimia, sepsis, or even death (Foxman 2002). Many factors are attributed to these infections such as bacterial invasion, anatomical and functional abnormalities of urinary tracts and host immune status. In terms of bacterial invasion, a various uropathogens have been identified as the common causative agents of these infections, namely *Escherichia coli, Klebsiella* sp.*, Proteus, Pseudomonas, Staphylococcus saprophyticus* and *Enterococcus* (Barnett and Stephens 1997). Of these uropathogenic bacteria, *Escherichia coli* is the most widely known accounting for more than half of UTI incidences (Kucheria 2005). These bacteria possess not only virulence factors but also antibiotic resistance in promoting and persisting colonization and infection of the urinary tract (Oelschlaeger 2002; Rijavec et al. 2006).

One of the antibiotic resistant features of *E. coli* that should be concerned is cephalosporinase, a class C β-lactamase enzyme that hydrolyses cephalosporins, as a result of increasing use of ceftazidime and other third-generation cephalosporins. This enzyme is encoded by *ampC* genes located on the chromosomes as well as plasmids (Philippon et al. 2002). The location of these genes in the plasmid potentially allows dissemination among different species by horizontal transfer (Su et al. 2008).

A clone of antibiotic uropathogenic *E. coli* in the northwest of England that its entire genome sequence has been obtained, namely ST 131, carried these genes on its plasmids (Su et al. 2008). Certainly, an examination of the occurrence of the plasmid is crucial since it may be disseminated through horizontal transfer among bacterial populations leading to an increase of the prevalence of cephalosporinases within the strains.

The aim of the research is to isolate as well as to purify large plasmids containing cephalosporinase conferred by a panel of uropathogenic *E. coli* isolates. Restriction enzyme digestion of the large plasmids will be conducted to compare the restriction profile of such isolates, as this will allow a deeper understanding of the relationships of the isolates.

MATERIALS AND METHODS

Bacterial isolates

A total of 36 routine clinical *E. coli* isolates designated ST 131 appearing to be resistant to extended-spectrum cephalosporins were used in this study. The source of the isolates were from laboratories across the northwest of England, namely Manchester (isolate 1, 2, 25-32), Preston (isolate 3-6, 13-24 and 33-36), Lancaster (isolate 7 and 8)

and Barrow (isolate 9-11). Those isolates were then sub-cultured on nutrient agar and incubated at 37°C overnight. The antimicrobial susceptibility of each isolate was determined by Vitek 2 system by using the AST-N054 card (BioMerieux). For plasmid isolation purposes, the isolates were inoculated into Luria-Bertani broth followed by aerobic incubation at 37ºC for 18 hours. Before harvesting the cells, the optical density of the culture were measured by spectrophotometer (Cecil Instruments Ltd., Cambridge, UK) at wavelength 600nm. The number of cells was confirmed by total plate count.

Plasmid isolation

Plasmids were isolated by using Wizard® plus SV minipreps DNA purification system (Promega, Madison, USA) according to manufacturer's instruction. Briefly, an each overnight culture of *E. coli* in 1.5 mL LB broth was pelleted in sterile 1.5 mL eppendorf tube by centrifugation at 10.000g for 5 minutes at room temperature. The cells were then resuspended thoroughly and plasmid isolation performed. Plasmid DNA was eluted from spin columns by adding 100µl of nuclease free-water and centrifuging at 14.000g for one minute at room temperature. The eluted plasmid DNA was stored at -20ºC or run in a gel electrophoresis.

Gel electrophoresis

Gel electrophoresis was performed as described (Voytas 2000). The gel (5 mm thick) containing 0.7% of agarose was used in electrophoresis to resolve the isolated plasmid. In order to facilitate visualization of DNA fragments during the run, 0.5µg/mL of ethidium bromide was added before pouring the melted agarose to the gel-casting platform. After loading the DNA sample and Lambda DNA/EcoR I + HindIII marker (Promega, Madison, USA) mixed with 0.03 mL of 5x loading buffer (Bioline Inc., London, UK), the gel was run in 1x TBE buffer (in g/l: Tris, 10.8; EDTA, 10.3; boric acid, 5.5, pH 8.0) at 10V/cm for 75 minutes. Photographs of the gels followed by molecular weight measurements were taken by video imaging systems (Alpha Innotech Corp., San Leandro, California, USA).

Restriction enzyme analysis

A set of large plasmids from selected isolates were digested by using *Hpa*I restriction enzyme. For analysis, the enzyme was diluted by mixing 10µl of RE (10u/µl) and 40 µl of 1x RE buffer (buffer J). In a 0.5 mL sterile eppendorf tube, 2µl of 10x buffer J and 0.2µl of acetylated BSA were mixed gently by pipetting. A total of 15.3µl of plasmid DNA sample was then added to the suspension before adding 2.5µl of diluted restriction enzyme yielding a final volume 20µl. The tube was then centrifuged for a few second followed by incubation at 37 ºC for four hours. After adding 3µl of 5x loading buffer, the sample was loaded in agarose gel for electrophoresis.

Pulsed Field Gel Electrophoresis (PFGE)

The PFGE was conducted as describe previously (Sambrook and Russel 2001) with modification. Briefly,

the PFGE was performed in four steps, namely preparation DNA plugs, washing, electrophoresis and gel staining. Initially, an each overnight selected culture of *E. coli* in 9 mL of LB was centrifuged at 3,400g for 15 minutes. The supernatant was removed followed by adding 10 mL of TBE buffer and re-centrifuged for 15 minutes at 3.400g at room temperature. The supernatant was once again discarded and 0.5 mL of suspension buffer was added. The bacterial suspension was then gently mixed with 0.75 mL of melted 3% low melting point agarose (maintained at 50ºC) and quickly pipetted into the slots of a plastic plug mould (BIORAD). The plugs were allowed to solidify at 4 ºC for one hour before they were transferred to a sterile glass bijou containing 1 mL RNAse (50µg/mL) followed by incubation at 37ºC for 4 days. RNAse was subsequently replaced by lyses buffer (500µg/mL lysozyme solution) before the plugs were incubated overnight at 37ºC. Having been incubated overnight, the lyses buffer was replaced with 1 mL proteolysis buffer (1% SDS solution) followed by adding proteinase K solution (1mg/mL) per sample. For the digestion to take place, the plugs were incubated overnight at 55ºC at a shaking water bath before the next step, washing, was carried out.

The washing step was performed six times every hour at room temperature by replacing the proteinase K solution with 2 mL TE buffer in order to eliminate cell debris and proteinase K activity as well as to equilibrate the agarose plugs. The washing step was carried out carefully without disturbing the plugs. After washing, the plugs were stored at 4ºC prior to using in further steps.

In the third step, the DNA plugs were sliced and loaded into appropriate wells of 1.2% agarose gel and sealed with 3% low melting point agarose. A DNA molecular weight marker plug (Sigma-Aldrich, Inc, Missouri, USA) was also loaded in one of the wells. The gel was then placed in electrophoresis chamber of a CHEF Mapper System (BIO-RAD Laboratories Ltd., Hemel Hempstead, UK) that had been poured with 2 liters of 0.5x TBE buffer. The system was run with parameters: initial switch time, 6s; final switch time, 8s; run time, 24h, angle, 120º; gradient, 4.5V/cm; temperature, 14ºC; ramping factor, linear. Ultimately, in the last step, ethidium bromide (0.5 µg/mL in water) and distilled water were used to stain and wash the gel respectively for 20 minutes for each step followed by visualization of stained gel under UV light with video imaging systems.

RESULTS AND DISCUSSION

Colonial morphology and cell density

A total of 36 *E. coli* ST 131 isolates grew well on the nutrient agar showing typical colonial appearance with diameter 2-4mm, circular, slightly convex, gray and moist. Incubation overnight at 37ºC in LB yielded cell numbers of *E. coli* of 3.7×10^9 colonies with optical density at 600 nm (OD600) of 0.803.

LB used in this project has given a profound effect to allow fast growth and good growth yield of *E. coli* to reach exponential state of bacterial growth. LB is a rich medium

containing catabolizable amino acids as carbon sources accounting for the alkalinisation of the medium during growth. It is important to note that exponential growth of culture in plasmid isolation is crucial since in this rate, the plasmids of *E. coli* increase rapidly due to replication process in parallel with cell division every 20 minutes (Sezonov et al. 2007). Furthermore, improper time and temperature incubation will yield relatively low amount of plasmid DNA because of insufficient density of bacterial cells. Likewise, overgrown cultures may also result in suboptimal yields as well as impurity yields due to excessive chromosomal DNA contamination as part of autolysis of bacterial cells after reaching stationary phase (Promega 2008)

Antimicrobial susceptibility

Antimicrobial agent susceptibilities of *E. coli* ST131 used in this study is listed in Table 1. It is worth noting that a total of 36 *E. coli* ST131 isolates, phenotypically, showed resistance to penicillins such as ampicillin, amoxicillin-clavulanic acid and piperacilin with rates approximately 89%, 67% and 80% respectively (Table 1). These isolates were also resistant not only to cefalotin (73%), a first generation cephalosporins, but also to quinolone drugs such as nalidixic acid with rates over 83% and ciprofloxacin with rates of 80%. Likewise, the rate of trimethoprim resistance of these isolates was also considerably high (80%).

Plasmid isolation

Of the 36 isolates of *E. coli* ST 131, eight of them harbored a large plasmid, namely isolate 9 (Figure 1; lane c), 10 (lane d), 12 (lane e), 17 (lane g), 18 (lane h), 26 (lane k) and 30 (lane m) with molecular weight approximately

above the chromosomal DNA artifacts (~21kb). Based on the migration patterns, those large plasmids were classified as plasmid w (~42kb), plasmid x (~40kb), plasmid y (~30kb) and plasmid z (~28kb). Isolate 10 possesses both plasmid w and z, whereas isolate 9, 12 and 17 possess plasmid w only. The remaining isolates which are isolate 18, 26 and 30, posses plasmid x, y and z, respectively.

In this agarose gel, small plasmids, possibly with three bands were also detected. Conversely, in particular isolates, there were only chromosomal DNA band that had been detected instead of plasmid i.e. isolate 8 (lanes b), isolate 14 (lanes f), isolate 19 (lanes i), isolate 20 (lanes j) and isolate 27 (lanes l). The resolution of those isolates on the agarose gel is shown in Figure 1.
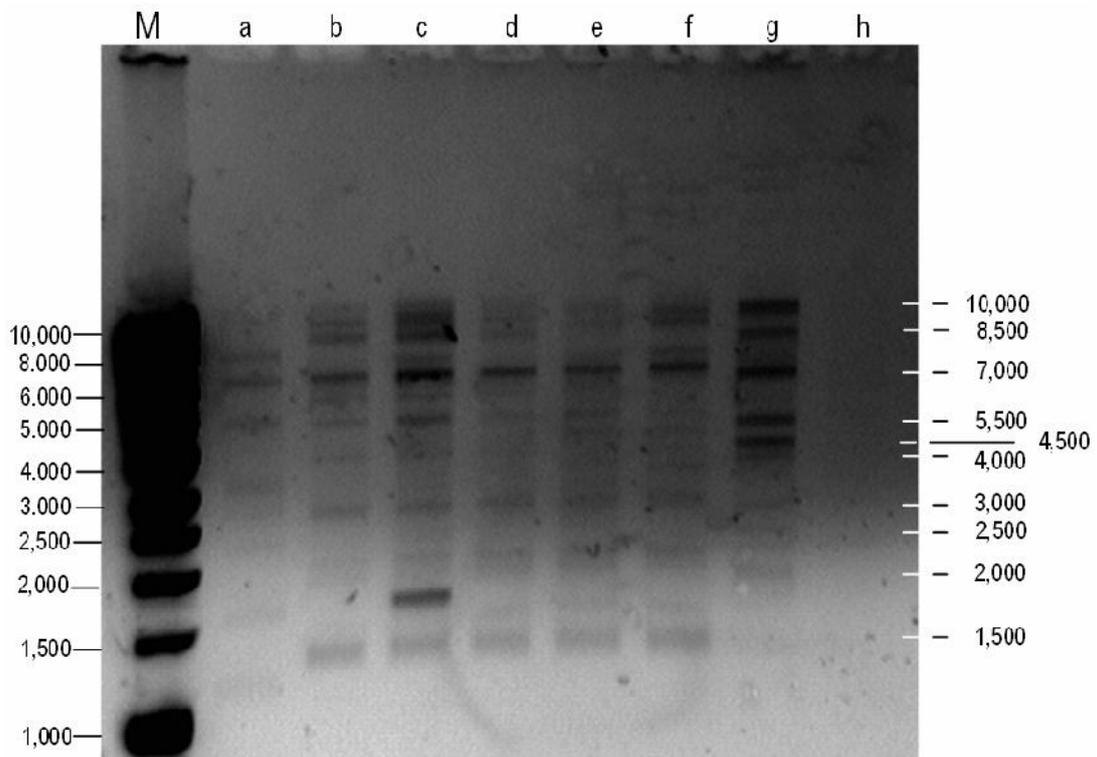
Restriction enzyme analysis

Activity of *Hpa*I is most likely active to digest almost all of the plasmid of *E. coli* isolates harboring large plasmids yielding some restriction fragments. It is apparent that isolate 9, 10, 12, 17 and 18 have the common restriction patterns yielding 10.000bp, 7.000bp, 5.000bp, 2.750bp, 2.000bp and 1.400bp fragments as shown in Figure 2 lanes b, c, d, e and f respectively. These indistinguishable fragments may indicate that the plasmids among those isolates are closely related. On the other hand, *Hpa*I digested the large plasmid of isolate 26 yielding quite distinct fragments than the previous isolates, namely in base pairs 10.000, 8.500, 7.000, 5.500 and 4.500. The most striking feature of this restriction enzyme digestion, however, was there is no restriction activity on the plasmid of isolate 30 as indicated by no resolved fragments on the gel (lanes h).

Table 1. Summarized susceptibilities of *E coli* ST131 (*n* = 36) against antimicrobial agents.

Antimicrobial agents	Resistant *n* (%)		Intermediate *n* (%)		Susceptible *n* (%)		Total
Ampicillin	32	(88.89)	0	(0)	4	(11.11)	36
Amoxicillin/Clavulanic Acid	24	(66.67)	2	(5.56)	10	(27.78)	36
Piperacillin	29	(80.56)	3	(8.33)	4	(11.11)	36
Piperacillin/Tazobactam	0	(0)	14	(38.89)	22	(61.11)	36
Cefalotin	26	(72.22)	5	(13.89)	5	(13.89)	36
Cefuroxime	26	(72.22)	0	(0)	10	(27.78)	36
Cefuroxime Axetil	26	(72.22)	0	(0)	10	(27.78)	36
Cefoxitin	22	(61.11)	0	(0)	14	(38.89)	36
Cefotaxime	8	(22.22)	18	(50)	10	(27.78)	36
Ceftazidime	2	(5.56)	24	(66.67)	10	(27.78)	36
Cefepime	1	(2.78)	25	(69.44)	10	(27.78)	36
Aztreonam	1	(2.78)	25	(69.44)	10	(27.78)	36
Lmipenem	0	(0)	0	(0)	36	(100)	36
Meropenem	0	(0)	0	(0)	36	(100)	36
Ertapenem	0	(0)	0	(0)	36	(100)	36
Amikacin	0	(0)	17	(47.22)	19	(52.78)	36
Gentamicin	5	(13.89)	1	(2.78)	30	(83.33)	36
Tobramycin	16	(44.44)	1	(2.78)	19	(52.78)	36
Nalidixic Acid	30	(83.33)	0	(0)	6	(16.67)	36
Ciprofloxacin	29	(80.56)	0	(0)	7	(19.44)	36
Nitrofurantoin	0	(0)	1	(2.78)	35	(97.22)	36
Trimethoprim	29	(80.56)	0	(0)	7	(19.44)	36
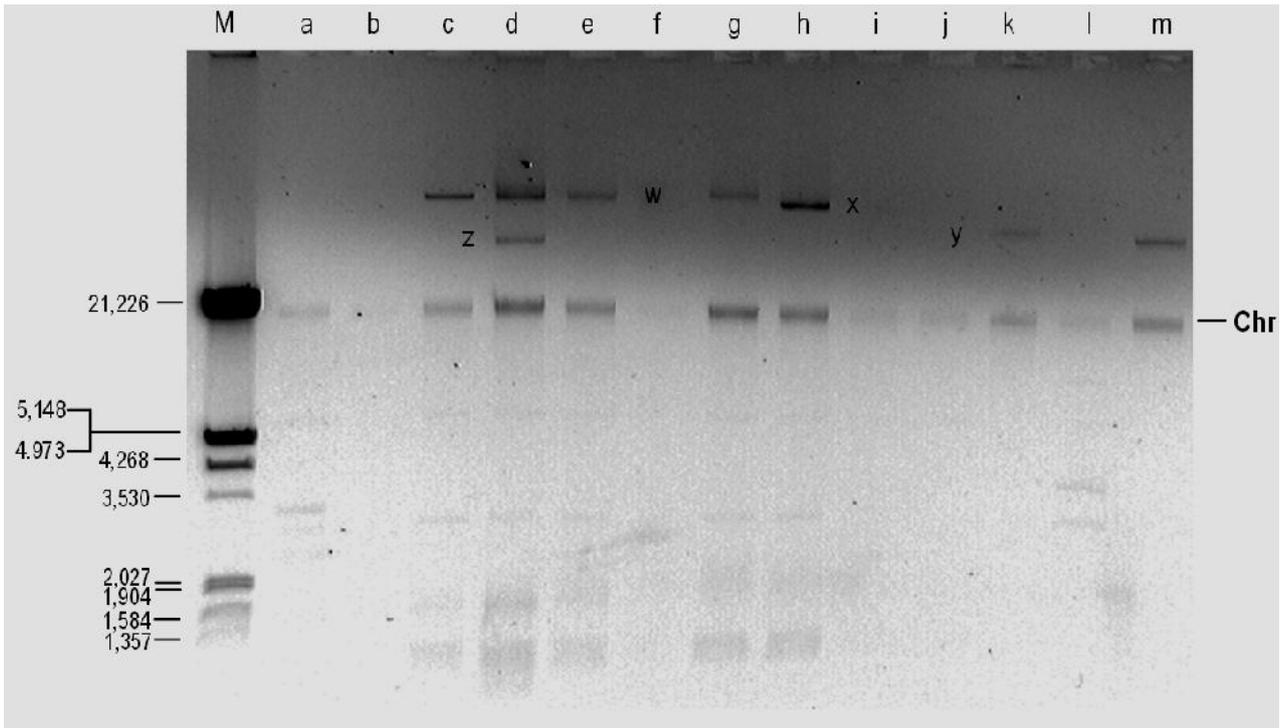
Figure 1. The resolution of some *E. coli* ST 131 plasmids on the 0.7% agarose gel run at 10V/cm. (M) Lambda DNA/*Eco*R I + *Hind*III markers, (a) isolate 3, (b) isolate 8, (c) isolate 9, (d) isolate 10, (e) isolate 12, (f) isolate 14, (g) isolate 17, (h) isolate 18, (i) isolate 19, (j) isolate 20, (k) isolate 26, (l) isolate 27 and (m) isolate 30. The large plasmids with their bands above the chromosomal (chr) artifacts (~17kb) were grouped as plasmid w (~42kb), plasmid x (~40kb), plasmid y (~30kb) and plasmid z (~28kb). Numbers indicate base pairs (bp).

Figure 2. The restriction profiles of *E. coli* ST131digested by *Hpa*I restriction enzyme on the 0.7% of agarose gel run at 10V/cm for 75 minutes. (M) molecular marker of Hyperladder I (Bioline, UK), (a) isolate 3, (b) isolate 9, (c) isolate 10, (d) isolate 12, (e) isolate 17, (f) isolate 18, (g) isolate 26 and (h) isolate 30. Numbers indicate base pairs (bp).

*Hpa*I used in this study is an endonuclease enzyme recognizing as well as digesting in the middle of double stranded DNA at the sequence 5'- GTT   AAC -3' leaving blunt ends (Ito et al. 1992). Based on the patterns of restriction fragments in Figure 2, isolate 9, 10, 12, 17 and 18 are likely to be closely related as these isolates share the same digestion patterns. Compared to previous works, the restriction patterns of these isolates also shared the similar restriction profile with pTcTN49 (Lavollay et al. 2006), but quite different from the profile of pC15-1a (Boyd et al. 2004). Plasmid of pTcTN49 is a plasmid harboring CTX-M-15 that has clonally disseminated in Paris (France), Tunis (Tunisia) and Bangui, Central African Republic (CAR), whereas pC15-1a has disseminated in Toronto, Canada. Isolate 26 and 30, on the other hand, tend to be different strains with the previous isolates as these isolates possessed quite distinguishable digested fragments or no fragments at all respectively. However, the restriction pattern of isolate 26 shared the same profile with pEpTU (Lavollay et al. 2006).

Pulsed Field Gel Electrophoresis

PFGE patterns of selected *E. coli* ST131 isolates harboring large plasmids comprised various profiles, designated a-h (Figure 3). Three large plasmids were detected in both isolate 10 (lanes c) and isolate 17 (lanes e). The descending molecular size in of three copies of large plasmids in isolate 10 were ~92kb, ~45kb and ~28kb as opposed to ~118kb, ~46kb and ~33kb of isolate 17 respectively. Two plasmids were detected in five isolates, namely isolate 9 (lanes b), isolate 12 (lanes d), isolate 18 (lanes f), isolate 26 (lanes g) and isolate 30 (lanes h), whereas single plasmid was only detected in isolate 3 (lanes a).

The presence of large plasmids in *E. coli* ST131 isolates conferring antibiotic resistance may allow ST131 confer virulence genes as well since plasmids encoding adaptive trait genes certainly preceded antibiotic resistance, may contain genes with a role in bacterial colonization and virulence (Martinez and Baquero 2002). These genes are encoded as a response of a local transient adaptation to a wide range of hosts and are able to be fine-tuned in order to allow a degree of genetic flexibility, which would not be available if the relevant genes were located on the chromosomes (Summers 1996). These plasmid-encoded virulence genes have been implicated in adhesions, toxinogenesis, serum resistance and cell invasiveness (Martinez and Baquero 2002).

It is imperative to note that the plasmids may also confer plasmid transfer genes to promote the virulence dissemination among bacterial populations. Chen et al. (2006) reported plasmid of uropathogenic *E. coli* UTI89, namely pUTI89, share conjugative genes and several virulence genes indicating a role of pathogenesis. pUTI89 encodes *tra* operon involved in conjugative DNA transfer, whereas virulence genes such as *cjrA*, *cjrB*, *cjrC* and *senB* coding for iron uptake proteins and enterotoxin.
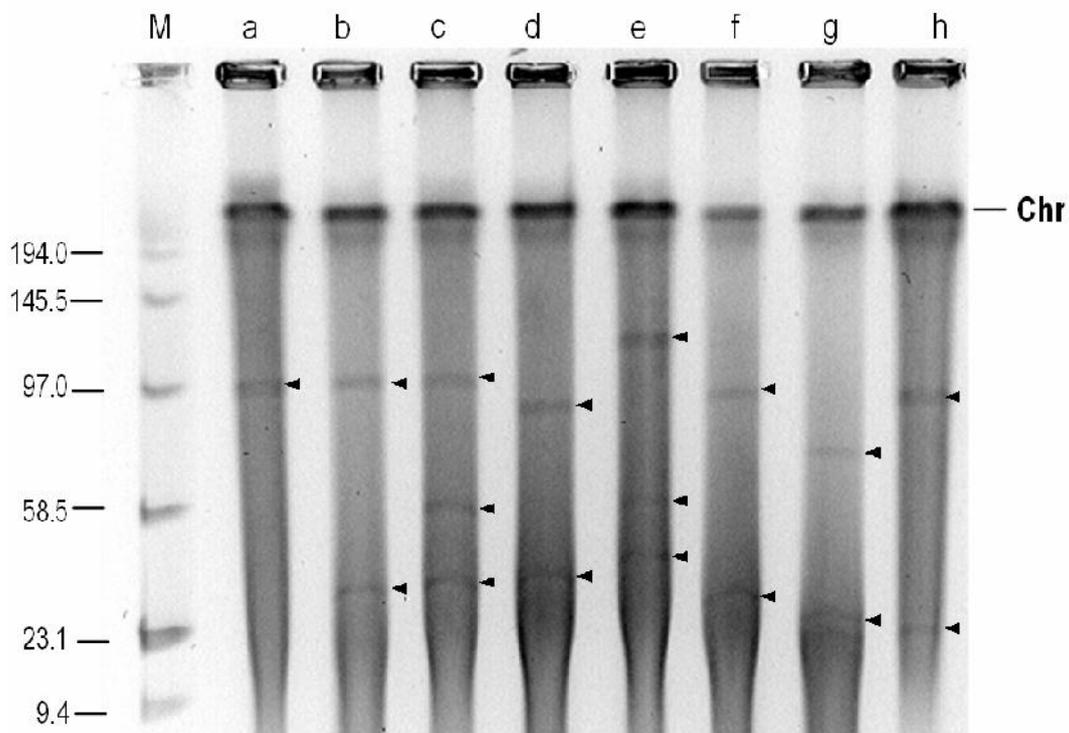
Figure 3. Plasmid bands (triangle) resolved by PFGE of selected *E. coli* ST131 isolates. (M) molecular marker, (a) isolate 3, (b) isolate 9, (c) isolate 10, (d) isolate 12, (e) isolate 17, (f) isolate 18, (g) isolate 26 and (h) isolate 30; Chr, chromosomal DNA. Numbers indicate kilo-base pairs (kb).

CONCLUSIONS

Of 36 isolates of *E. coli* ST131 showing high resistance to penicillins, cephalosporins and trimethoprim, large plasmids have been isolated and detected in eight isolates, namely isolates 3, 9, 10, 12, 17, 18, 26 and 30 with the largest molecular size was ~42kb confirmed by agarose gel electrophoresis and ~118kb by PFGE. There might be close relation among those isolates owing to the common restriction patterns in restriction enzyme analysis. Both agarose gel electrophoresis and PFGE are considerably useful not only to resolve the isolated large plasmids but also to measure their molecular size.

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