

Full Length Research Paper

# Molecular characterization of extended-spectrum beta-lactamase (ESBL) producing extra-intestinal pathogenic *Escherichia coli*

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Accepted 27 October, 2011

Despite the frequent isolation of extended-spectrum beta-lactamase producing *Escherichia coli* from extra-intestinal infections (ESBL-ExPEC), the virulent properties or the antibiotic resistance mechanisms or its phylogenetics is rarely studied in Malaysia. In the present investigation, we characterized 95 clinical isolates of ESBL-ExPEC collected over a one year period time from a tertiary hospital through Clermont dichotomous phylogenetic grouping, virulent gene profiling and ESBL production mechanisms. The highest frequency of *E. coli* isolation was observed among urine (40% / n=38), while the lowest was among tissue and peritoneal dialyses fluid (1.05% / n= 1). Phylogenetic grouping revealed that 42 (44.21%) isolates belonged to group D, while 26 (27.36%), 14(14.73%) and 13 (13.68) belonged to A, B2 and B1. All isolates were multi drug resistant, whereby 58.9% harboured CTX-M-1 and 22.10% CTX-M-9. None of the isolates carried CTX-M-2 or CTX-M-8 clusters. Eight (7.6%) isolates were positive for bla<sub>TEM-1</sub> and two (1.9%) produced SHV-12 beta-lactamase. The CTX-M-9 cluster was found to be significantly (P=0.005) associated with pathogenic group. Majority of the isolates (48.42%) carried class 1 integrons, while 3 (3.15%) isolates showed positive for class 2-encoded *intI2* integrase and no signal for class 3 integron. Virulence factors profiling identified *iutA* 56 (58.94%) and *kpsMII33* (34.73%) as frequent pathogenic determinants. Virulence studies on *Caenorhabditis elegans* showed that antibiotic susceptible *E. coli* strains have short life span than pathogenic or commensal ones. In conclusion, it is found that commensals are becoming multiple drug resistant and carries several virulent genes which warn the spread of drug resistance or virulent factors to the normal flora.

**Key words:** *Escherichia coli*, ESBL, ESBL-ExPEC, bla<sub>TEM</sub>, bla<sub>SHV</sub>, CTX-M, *Caenorhabditis elegans*, Malaysia.

## INTRODUCTION

*Escherichia coli* are one of the commensals in the human intestinal tract. As a commensal, it contributes to the maintenance of the health of a person. However, when *E. coli* enters into unnatural sites, it causes variety of infectious diseases (Mendonca et al., 2007). *E. coli* are classified into three major groups; commensal, intestinal (enteric or diarrheagenic) and extra-intestinal pathogen

(ExPEC). Commensal offers benefits to the host by resisting colonization by pathogens (Donnenberg, 2002). Intestinal *E. coli* causes infections in the gastro-intestinal tract leading to enteritis, enterocolitis, and colitis. Extra intestinal *E. coli* strains becomes pathogenic when it enters from its colonization site to multiple other anatomical sites such as urinary tract, bloodstream, cerebral spinal fluid, respiratory tract, and peritoneum (spontaneous bacterial peritonitis), resulting in infections (Diard et al., 2007). Hence, extra intestinal *E. coli* is generally termed as ExPEC. Since ExPEC has the capability to enter multiple anatomical sites, the host

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environment and associated defense mechanisms varies, resulting in having distinctive virulence trait. The virulence determinants present in most ExPEC include various adhesions (for example, P and type I fimbriae), factors that avoid or subvert host defense systems (e.g. capsule, lipopolysaccharide), mechanisms for nutrient acquisition (for example, siderophores), and production of toxins (for example, hemolytic, cytotoxic necrotizing factor 1) (Mamlouk et al., 2006).

Phylogenetic analysis based on Clermont dichotomous tree has shown that *E. coli* strains fall into four main phylogenetic groups: (A, B1, B2, and D) (Clermont et al., 2000). Most of the virulent extra intestinal strains are derived from group B2 and, to a lesser extent from group D whereas most commensal strains derive from groups A and B1 are with low virulence properties (Ho et al., 2007 and Lim et al., 2009). Group B2 is more commonly detected among non immunocompromised patients, while groups A and B1 frequently isolated from immunocompromised patients (Ho et al., 2007). Distribution of virulence factors in clinical strains varies based on the phylogenetic groups and on the type of infections (Karisik et al., 2008)

In a clinical setting, *E. coli* infections are treated with beta-lactamase, whose extensive use has led to emergence of extended-spectrum beta-lactamses (ESBLs) producing strains. ESBLs are enzymes produced by Gram-negative bacilli that have the ability to hydrolyze beta-lactamses antibiotics containing an oxyimino group. ESBLs are classified into nine distinct structural /evolutionary families based on their amino acid sequences, among them SHV, TEM and CTX-M form the major families. The recently emerged CTX-M family which contains more than 40  $\beta$ -lactamses are grouped into five distinct clusters such as CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Bonnet, 2004).

Earlier studies carried out on ESBLs in Malaysia have mainly focused on the different ESBL determinants among the clinical isolates (Lim et al., 2009; Song et al., 2009) and limits the data on the phylogenetics and virulence patterns. Therefore in the current study, ESBL *E. coli* collected from a public hospital in Malaysia over a one year period from January to December 2006 were investigated for the virulent determinants, types of ESBLs and its association with different phylogenetic groups.

## MATERIALS AND METHODS

### Clinical isolates

A total of 95 non-repetitive ESBL ExPEC strains isolated from different clinical sources (urine, pus, body fluid, blood, tissue, and peritoneal dialysis fluid) collected from a public hospital in Malaysia during January to December 2006 were investigated. All isolates previously species-identified in the hospital laboratory by standard methods including Gram staining, lactose fermentation on MacConkey agar, metallic sheen on Eosin methylene blue agar and indole and methyl red positive, Voges-Proskauer and citrate negative by IMViC test, were reconfirmed in our research laboratory

stationed at University Putra Malaysia.

### Antimicrobial susceptibility testing and ESBL detection

All isolates were tested for antibiotic susceptibility against seven antibiotics representing five classes of beta lactams such as penicillins (ampicillin 10  $\mu$ g), cephalosporins (cefotaxime 30  $\mu$ g, cefepime 30  $\mu$ g), carbapenems (imipenem 10  $\mu$ g), monobactams (aztreonam 30  $\mu$ g), beta lactamase inhibitors (ampicillin-sulbactam 20  $\mu$ g) and sulphonamides (trimethoprim-sulfamethoxazole 25  $\mu$ g) by the disk diffusion method and interpreted as per CLSI guidelines (CLSI, 2007) with ATCC *E. coli* 25922 as control. ESBL production was determined by CLSI disk diffusion method using ceftiaxone 30  $\mu$ g, ceftazidime 30  $\mu$ g, and aztreonam 30  $\mu$ g and double-disk approximation method (Jarlier et al., 1988)

### ESBL genes detection

PCR amplification of the *bla*SHV, *bla*TEM, *bla*-CTX-M-1, *bla*-CTX-M-2, *bla*-CTX-M-8, and *bla*-CTX-M-9 were performed as described previously (Rahman et al., 2004; Henriques et al., 2006). Boiled suspension of bacterial cells was used as DNA template (Rasheed et al., 1997). All amplified products obtained were sequenced to validate their identities. Clinical isolates of *E. coli* that were sequence confirmed for *bla*TEM, *bla*SHV and *bla*CTX-M were used as the positive controls. Positive controls for CTX-M-2, CTX-M-8 and CTX-M-9 were obtained from Antibiotic Resistance Monitoring and Reference Laboratory (ARMRL), London. Isolate positive for CTX-M-1 cluster was kindly provided as a gift by Professor Patrice Nordmann (Department of Bacteriology-Virology, Hospital Bicetre, and Paris).

### Detection of class 1, 2, and 3 integrons

Class 1, 2, and 3 integrons were detected by PCR as described previously (Oelschlaeger et al., 2002). Representative PCR products were sequenced to confirm their identities.

### Phylogenetic grouping

Phylogenetic grouping (A, B, B2, and D) of the *E. coli* isolates was determined with a PCR method (Clermont et al., 2000) based on the combination of three DNA markers such as *chuA*, *yjaA* and *TspE4.C2*. Briefly *chuA*-positive/*yjaA*-positive, *chuA*-positive/*yjaA*-negative, *chuA*-negative/*TSPE4.C2*-positive, and *chuA*-negative/*TSPE4.C2*-negative strains were classified as B2, D, B1, and A respectively.

### Virulence genotyping

All ESBL *E. coli* isolates were tested by PCR for the presence of a panel of virulent genes as described (Arisoy et al., 2008) using template DNA prepared by boiling method (Ruppe et al., 2009). The virulent genes included *iutA* (aerobactin iron transport system), *kpsMTII* (capsular polysaccharide), *hlyA* (hemolysin) *sfa/foc* (S fimbriae and F1C fimbriae), *afa/draBC* (Dr family adhesin) and *Pap* operon (pyelonephritis-associated Pili).

### *Caenorhabditis elegans* nematode killing assay

The *Caenorhabditis elegans* infection assay was performed as described previously (Diard et al., 2007). The nematodes and *E. coli* strain OP50 (an avirulent control strain) were provided by

**Table 1.** Distribution of CTX-M types among phylogenetic groups of *E. coli*.

Group (No)	CTX-M-15	CTX-M-3	CTX-M-55	CTX-M-79	CTX-M-33	Total of CTX-M-1	CTX-M-9	CTX-M-14	CTX-M-24	CTX-M-65	CTX-M-27	Total of CTX-M-9
A (26)	11	5	0	1	1	18	1	2	0	0	0	3
B1 (13)	2	2	2	0	0	6	0	0	0	0	0	0
B2(14)	7	1	0	0	0	8	1	5	0	1	0	7
D (42)	19	4	1	0	0	24	0	6	3	0	2	11
Tot. (95)	39	12	3	1	1	56	2	7	3	1	2	21

J. Ewbank (CIML, Marseille, France). Briefly 100 µl of test bacterial culture was spread on the 9-cm-diameter plates containing NGM agar (Sutphin and Kaerberlein, 2009). NGM media was prepared with 4% of agar instead of recommended 1.7% for the nematodes pick up. The plates were seeded with 20 L4-stage synchronized nematodes and incubated at 25°C. The dead worms were scored every 24 h for 15 days, while the lives ones were transferred to new NGM plate containing fresh bacterial lawn every 48 h. At least three replica experiments, repeated three times, were performed for each test isolate. A nematode was considered dead when it failed to respond while the plates were tapped or by a gentle touch with the platinum wire.

## RESULTS

Among the 95 ESBL *E. coli* isolates studied, the highest frequency was observed among urine (40% / n=38), followed by pus (29.47% / n=28), blood (18.94% / n=18), body fluids (9.47% / n=9%), while the lowest was seen among tissue and peritoneal dialysis fluid (1.05% / n= 1). Phylogenetic grouping revealed that 42 (44.21%) isolates belonged to group D, while 26 (27.36%), 14(14.73%) and 13 (13.68) belonged to A, B2 and B1.

### Antibiotic resistance and phylogenetic characterisation of *E. coli* isolates

The antibiotypes of the ESBL producing *E. coli*

were as follows: 95(100%), 78(82.10%), 77(81.05%), 70(73.7%), 53(55.78%) of the isolates were resistant to ampicillin, cefotaxime, trimethoprim/sulfamethoxazole, aztreonam and cefepime. All isolates were susceptible to imipenem.

PCR analysis showed that the majority of the ESBL isolates are CTX-M beta lactamases producers (Table 1). Fifty-six (58.9%) of 95 ESBL isolates harboured CTX-M-1 cluster which contained CTX-M-15 41% (39), CTX-M-3 12.63% (12), CTX-M-55 3.15% (3), CTX-M-33 and CTX-M-79 1.05% (1 each), while 21 (22.10%) were CTX-M-9 clusters containing CTX-M-14 13.68% (13), CTX-M-24 3.15% (3), CTX-M-9 and CTX-M-27 2.10% (2 each), and CTX-M-65 1% (1). None of the isolates harboured CTX-M-2 or CTX-M-8 clusters. Eight isolates harboured bla<sub>TEM-1</sub> of which two had CTX-M-1 types (CTX-M-15 and CTX-M-3). Of the 95 ESBL *E. coli* isolates only two (1.9%) produced SHV-12 beta-lactamase. The CTX-M-9 cluster was significant (P=0.005) among pathogenic (B2 and D) than commensal groups (A and B1), where majority of them belonged to group D (52.38%) and 33.33% to group B2. CTX-M-1 cluster did not show any significant association (P=0.668) with either pathogenic (B2: 8/14.28%; D: 24/42.85%) or commensal group (A: 32.14%; B1:6/10.7%). TEM (B2:0; D: 4/50%; A: 2/25%; B1: 2/25%) and SHV (B1: 2/100%) did not show any significant association (P > 0.05) with phylogenetic groupings (Table 4).

### Class 1, 2, and 3 integrons

Among the ninety-five ESBL *E. coli* isolates screened for the presence of integrases encoded on class 1, 2, and 3 integrons, the class 1 integron-encoded *intl1* integrase gene was detected in 46 (48.42%) isolates while 3 (3.15%) isolates tested positive for class 2-encoded *intl2* integrase. None of the isolates showed positive signal for class 3 integron.

### Virulence genotypes

PCR analysis for virulence factors showed that 2 (2.10%), 56 (58.94%), 7 (7.36%), 2 (2.10%), 1 (1.05%) and 33 (34.73%) of the isolates carried the *hlyA*, *iutA*, *sfa/foc*, *afu/dra*, *pap* and *kpsMIII* genes.

### Pathogenicity of *E.coli* on *Caenorhabditis elegans*

In order to identify the associations between virulence factors and pathogenic potential among commensal and pathogenic strains, one isolate each from pathogenic (B2) and commensal (A) group containing a panel of virulent genes such as *kpsMIII*, *iutA* and *sfa/foc* were tested on *C. elegans*. In addition, to determine whether the sensitive or resistant strains are pathogenic, a

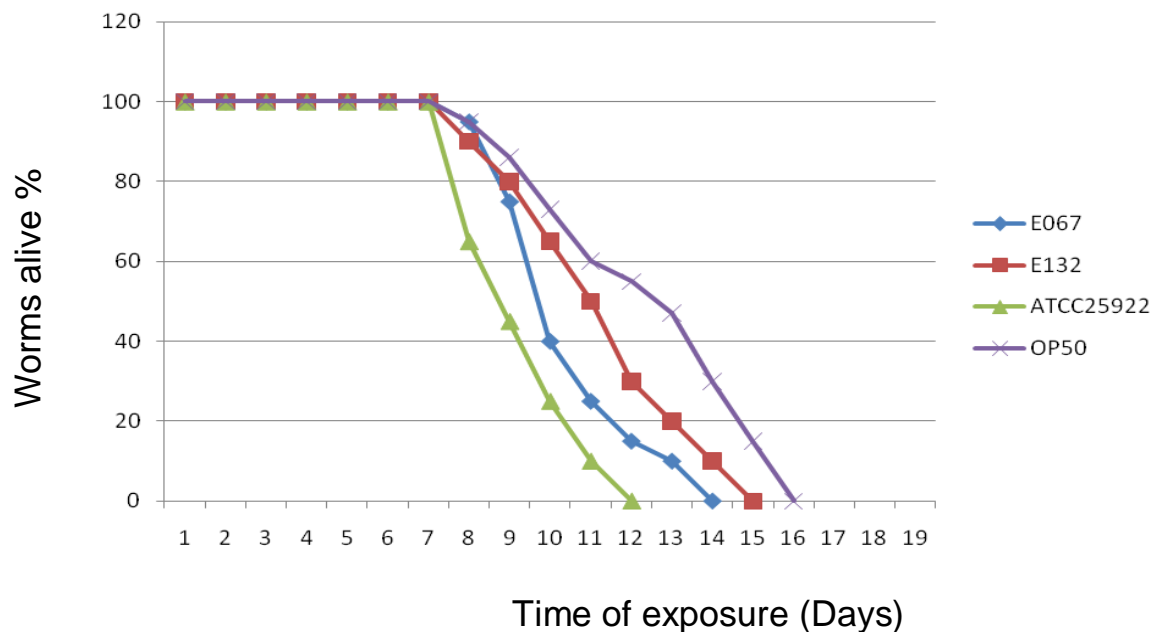


Figure 1. Survival curve of *C. elegans* fed with different strains of *E. coli*.

Table 2. Distribution of virulence factors among phylogenetic groups of *E. coli* strains.

Group	<i>kpsMIII</i>	<i>iuta</i> (%)	<i>Sfa/foc</i> No. (%)	<i>Afa/dra</i>	<i>Hly<sub>a</sub></i> (%)	<i>Pap</i> (%)
A	6 (18.18)	16 (28.57)	2 (28.57)	0	2 (100)	1 (50)
B1	3 (9.090)	4 (7.142)	0	0	0	0
B2	5 (15.151)	10 (17.857)	2 (28.571)	0	0	1 (50)
D	19 (57.575)	26 (46.428)	3 (42.857)	0	0	0

non-ESBL, drug sensitive *E. coli* (ATCC 25922) containing all three virulent genes was included in the investigation. *E. coli* OP50 which is used as the food source for *C. elegans* was used as a test control. All isolates tested showed virulence in the *C. elegans*, however the susceptible (ATCC 25922) took shorter time (12 days) compared to pathogenic (14 days) and commensal (15 days) ESBL strain. On the other hand, the death of *C. elegans* with *E. coli* OP50 was seen only after 16 days (Figure 1).

#### Comparative analysis of virulence and antibiotic resistant genotypes, and phylogenetic grouping

The virulence gene profile for different phylogenetic groups is listed in Table 2. The pathogenic group D contained most of the virulent genes. Group A, which is a commensal also carried high percentage of virulence genes indicating that even commensal strains could be pathogenic. Although resistant strains are known to be less pathogenic, virulent genes *kpsMIII* and *iutA* was found to be present in more than 25% of the ESBL

isolates producing different types of beta-lactamases. The virulent gene profile for different beta-lactamase producers are listed in Table 3.

#### DISCUSSION

Combating the emergence of ESBL *E. coli* is the greatest challenge to the clinicians as well as the infection control unit in a clinical setting. Data that is available from the routine laboratory diagnosis in a hospital only provides information on whether the isolate is an ESBL producer or not. For the efficient management of the ESBL-*E. coli* infections, information on the phenotypes, virulent properties and the antibiotic resistance types are highly necessary. Therefore, in the current study, the combination of phenotypic and genotypic methods has been applied to characterize the ESBL producing *E. coli* isolates isolated from clinical specimens.

As *E. coli* is the most frequent etiologic agent of urinary tract infection (Sutphin and Kaerberlein, 2009) it is not surprising to observe highest rate of isolation from urine (40%) in the current study. However, our results are

**Table 3.** Association between resistance genes and virulence factors.

Variable	CTX-M-1(%)	CTX-M-9 (%)	TEM (%)	SHV
<i>kpsMIII</i>	14(25)	8 (38.095)	3 (37.5)	0
<i>iutA</i>	29(51.785)	8(38.095)	3 (37.5)	0
<i>sfa/foc</i>	1 (1.785)	4(19.047)	2(25)	0
<i>hlyA</i>	0	1 (4.762)	0	0
<i>pap</i>	0	1(4.762)	0	0
<i>afa/dra</i>	0	0	0	0

**Table 4.** The association between antibiotic resistance genes, virulence factors and phylogenetic groups.

Variable	Commensal groups (A+B1) No. (%)	Pathogenic groups (B2+D) No. (%)	No.	p
B-lactamase genes				
<i>CTX-M-1</i>	24 (42.86)	32 (57.14)	56	0.668
<i>CTX-M-9</i>	3 (14.29)	18 (85.71)	21	0.005*
Virulence factors				
<i>iutA</i>	20 (35.7)	36 (64.3)	56	0.205
<i>kpsMIII</i>	9 (27.3)	24 (72.7)	33	0.046*
<i>Sfa/foc</i>	2(28.6)	5 (71.4)	7	0.486
<i>hlyA</i>	2 (100)	0	2	0.087
<i>pap</i>	1(100)	0	1	0.228

\*p is significant at <0.05.

lower compared to Bangladesh (48.09%) and Spain (50.3%) (Oelschlaeger et al., 2002; Piatti et al., 2007) as the rate of *E. coli* in UTI fluctuate widely depending on the local environment

All 95 ESBL *E. coli* isolates analyzed in this study were susceptible to imipenem. This finding is in agreement with previous reports (Ho et al., 2008 and Mamlouk et al., 2006). However, the isolates studied herein showed high rates of resistance to most of other antibiotics tested. The resistance rates of trimethoprim/sulfomethaxazole, aztreonam and ampicillin were much higher than the study reported in Malaysia previously (Lim et al., 2009). Possible explanation could be that their investigation contained only three ESBL producers by double disc diffusion test. However our resistant rates are similar for ampicillin, but lesser for trimethoprim/sulfomethaxazole, cefepime and cefotaxime when compared with data from Spain (Orman et al., 2002). Resistance to most of the antibiotics confirms the multiple drug resistant properties of the isolates.

From the current investigation it is found that majority (78.9%) of the ESBL *E. coli* isolates were CTX-M producers. Members of CX-M-1 and CTX-M-9 clusters have repeatedly been reported in Asia (Hawkey, 2008) as what is observed in the current investigation as well. However, our results are in contrast to a previous study from Malaysia, who reported high prevalence of TEM-1(88%) rather than CTX-M (20%). During the last two decades, most of the ESBL found in *E. coli* and in

general, in Gram-negative bacilli, has been of TEM or SHV lineage, which could be due to the ability of some insertion sequence elements to mobilize and promote the expression of B-lactamase (Piatti et al., 2007). The fact that we did not find much TEM or SHV in our isolates is noteworthy as the TEM and SHV lineage are slowly being replaced by CTX cluster worldwide (Livermore et al., 2007). Among the CTX-M cluster, CTX-15 was the most dominant type of ESBL identified in the current study which is in agreement with Korea (Rasheed et al., 1997) but in contrast with China (2 of 325, 0.61%) and Taiwan (2 of 128, 1.56%), where CTX-14 is the most prevalent type (Warren et al., 1996; Yan et al., 2006). ESBL producing *E. coli* are usually sensitive to  $\beta$ -lactamase inhibitors but in the current study 81% of isolates showed resistance to ampicillin/sulbactam, but only 56 (58.9%) of these isolates harboured CTX-M-1. However, 21 (22.1%) of isolates harboured CTX-M-9. This finding indicates that there are other beta-lactamases responsible for the resistance to beta-lactamase inhibitors which were unfortunately not investigated in the current study. Combined production of CTX-M and OXA enzymes by *E. coli* has shown to increase resistance towards  $\beta$ -lactamase inhibitors (Mendonca et al., 2007).

Phylogenetic analysis was carried out to investigate the genetic backgrounds of the ESBLs whether any of the isolate belong to commensal group or are purely pathogens. The ExPEC isolates studied herein clustered into different phylogenetic groups D, A, B2, and B1 at

different proportions (44, 27, 15, and 14%). Several studies suggest that ExPEC are mostly derived from the B2 phylogenetic group and to a lesser extent from the D group. However, our results are in contrast, whereby most isolates belonged to group D which is considered to be less pathogenic than B2 (Arisoy et al., 2008; Oelschlaeger et al., 2002; Livermore et al., 2007; Johnson et al., 2009). Isolates that belong to B2 are observed less in the current study, this could be because B2 which is more pathogenic than D are usually sensitive to antibiotics which is shown in several studies that antibiotic resistant strains are less pathogenic (Bert et al., 2008; Mendonca et al., 2007; Mamlouk et al., 2006; Ho et al., 2008; Hawkey et al., 2008; Piatti et al., 2007). Johnson et al. (2003) showed *E. coli* strains that are resistant to antibiotics shifted towards phylogenetic groups A and D, and moved away from group B2 which is also observed in the present study where all isolates were multidrug resistant and clustered in group D and A. Studies have also shown diversity in phylogenetic groups for ExPEC based on the host. Most of the ExPEC isolates from animal origin belongs to commensal groups A and B1 while human origin isolates belong to pathogenic groups B2 and D (Johnson et al., 2008).

Analysis of virulent genes in ExPEC isolates in the current study showed that 27(28.3%) isolates carried two or more virulence genes, while most of the other isolates contained one (42.1%) or none (29.4%). Distribution of virulence factors in ExPEC also varies according to the phylogenetic groups; the highest proportion of virulence factors is expected in pathogenic groups and low among commensals. However, in our study we did not find a significant difference between pathogenic and commensal groups. The pathogenic groups B2 (85.7%; n=12/14) and D (76.1%; n=32/42) had only slightly higher percentage of virulence compared to commensal group A (73%), but B1 showed much lesser percentage (38.4%). The low number of virulence gene detection among the ExPEC isolates could be because these could have come from normal flora which is usually found in the intestine or would have lost the VFs after acquiring the resistance genes. Similar observation was reported (Arisoy et al., 2008) as 40% of ExPEC strains were negative for virulence factors including *pap*, *sfa/foc*, *afa/dra*, *hly*, *cnfl* and *aer*. As limited number of virulence genes was tested, it is not sure whether the isolates that were negative for any of the tested VFs harbor any other pathogenic determinants. In the evaluation to see whether commensal or pathogenic group containing the same set of virulent genes which have the similar pathogenic effects or not, *C. elegans* which is an established model for investigating virulence mechanisms of ExPEC was used. From the results it is found that either commensal or pathogenic, showed similar killing effects (14 to 15 days). But when the susceptible strain carrying the same set of virulent genes tested, the killing of *C. elegans* was faster (12 days). Our results are in agreement with previous report (Lavigne et al., 2006) which

demonstrated that antibiotic susceptible strains killed nematodes faster than TEM or CTX-M producing isolates. As described before (Lavigne et al., 2006). The resistant strain kills the nematode faster due to its intrinsic virulence than the virulent strain *E. coli* OP50 which takes long time to kill ExPEC is also observed in our study.

In conclusion, we found commensals among extra intestinal infections, with multiple drug resistant properties. Majority of the isolates belonged to CTX 1 and 9. Isolates that were susceptible was found to be more virulent. Commensal with multiple drug resistant properties containing several virulent genes warns the spread of drug resistance to the normal flora. Proper usage of antibiotics in the hospital and environment are to be strictly monitored for the prevention of pathogenic ESBL *E. coli* strains that are highly dangerous.

## ACKNOWLEDGEMENTS

We are grateful to Professor Patrice Nordmann (Department of Bacteriology-Virology, Hospital Bicetre, and Paris) for providing CTX-M-1 cluster positive isolate. We express our special thanks to J. Ewbank (CIML, Marseille, France) for providing the nematode *Caenorhabditis elegans* and *E. coli* strain OP50 strains through Dr. Cheah Yoke Kqueen (Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University Putra Malaysia). This work was supported by the University Putra Malaysia Research University Grant Scheme UPM-RUGS) through grant number 04-01-09-0795RU.

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