UNIVERSITI PUTRA MALAYSIA

IMPROVEMENT OF MOLECULAR METHODS FOR DETECTION OF PATHOGENIC ESCHERICHIA COLI

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By

NAGI AHMED ABDULLAH AL-HAJ

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Doctor Philosophy

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DEDICATION

To my mother who patiently awaited my return to Yemen after the completion of my studies but unfortunately she passed away. May Allah blessed her and accepted her in the high paradise with my father.

To my wife and our children, Ahmed, Sala and Hamzah. They provided a much needed balance in my life and were much more reasonable about the many, many lost weekends than I could possibly have hoped for. This dissertation is dedicated to them.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

IMPROVEMENT OF MOLECULAR METHODS FOR DETECTIONOF PATHOGENIC *ESCHERICHIA COLI*

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Chairman: Associate Professor Mariana Nor Shamsudin, PhD

Faculty : Medicine and Health Sciences

Diarrhea is one of the leading causes of illnesses and death among children in developing countries, where an estimated 1.3 billion episodes and 4 to 10 million deaths occur each year in children below 5 years of age. The common pathogens of diarrhea are diarrheagenic *Escherichia coli* (DEC), Group A rotavirus, *Shigella* spp, *Salmonella* spp, *Campylobacter*, and *Vibrio cholerae*. Microbiological insights including phenotypic and genotypic characterisation are valuable approaches with application in management and prevention of diarrheal outbreaks by *E. coli*.

In the present study, the Random Amplified polymorphic DNA (RAPD) fingerprinting technique allowed genetic diversity assessment of 25 *E. coli* isolates. Six out of 20 arbitrary primers namely, OPAE 4, 9, 10, 11, 12 and 18 produced DNA fingerprinting patterns providing the discriminatory power and the display of the potential epidemiological and diagnostic markers. A highly significant finding from the DNA fingerprinting is the display of a predominant band at a size of 308 bp when arbitrary

fingerprinting is the display of a predominant band at a size of 308 bp when arbitrary OPAE-10 primer was used. The band consistently was amplified in *E.coli* strains from all sources but not in other Gram positive or Gram negative control strains.

The identity of this band after Blast analysis of TOPO 2.1–cloned sequence is a gene designated as *secD*. The finding is highly valuable with genotypic application as the gene is small in size and gave 100% homology to *E. coli* protein-export membrane, protein *secD*. In addition the high percentage of similarity (100%) of the RAPD-OPAE-10 marker to a fragment of *E. coli* genome emphasized the reliability of the marker as species-specific marker. The membrane assay developed using the probe designed from marker sequence consistently showed positive signal for all strains of *E. coli* tested but not for other Gram-positive and Gram-negative control strains tested, indicating the specificity and the sensitivity of *secD* marker primers and probes in species-specific detection of *E. coli* isolates. Although the RAPD OPAE-10 primers is commercially available, the RAPD OPAE-10 primer as *E. coli* diagnostic marker has not been reported previously. Another worthy finding from the RAPD study of the present work is the genetic diversity, and clonal groupings of *E.coli* strains from five different sources, determined simultaneously. The genetic diversity exhibited through the DNA fingerprinting patterns is accordingly observed in the antibiotic resistance pattern. A total of 70 isolates from different sources showed variations in resistance patterns to the 10 antibiotics tested from 61.2% *E. coli* isolates retrieved for antimicrobial resistance profiling. Overall, tetracycline and kanamycin were the most commonly reported antimicrobial agent resistance (81.0%) followed by chloramphenicol (76.0%), gentamycin (72.0%), and ampicillin (73.0%). While, resistances to ciprofloxacin of

24.0% norfloxacin, 27.0% and cefutoxin, 40.0% were low in prevalence in all types of samples. The phenotypic based conventional biochemical identification method was performed in the study to confirm strain identity of all bacteria used in the research.

A genetic assessment of *E.coli* strain of various sources based on he *GAD* gene revealed another candidate gene for gene probe development. The 671bp gene has been reported to be homogeneously present in many *E. coli* strains but the present study illustrated the presences in *E.coli* strains of various sources, namely clinical, animal, sea water, river, and food. All *E. coli* isolates examined had the 671-bp and carried *GAD* gene. A membrane-based dot blot hybridization technique assay applying oligonuclotide probe designed from the *GAD* gene sequences was not only highly sensitive and specific for all *E. coli* isolates from different sources, but a rapid assay was optimized enabling the detection to be compacted in a very short time.

The genetic assessment study of *E.coli* strains enable the multiple PCR-based methods to be optimized for pathogenic strain determination. The contribution of six primer pairs specific for enterohemorrhagic *E. coli* (EHEC) *stx1* and *stx2* genes, enteropathogenic *E. coli* (EPEC) *bfp* and *eae* genes, enterotoxigenic *E. coli* (ETEC) *elt* gene, and enteroaggregative *E. coli* (EAEC) *EAST* gene can facilitate the rapid detection of these groups from different sources through a single tube PCR method.

In addition the virulence gene-based for pathogenic strains detection, another virulence determination Lipopolysaccharide (LPS) found in the Gram negative bacteria was applied for development of genera differentiation method. A simple biolysate-based

method for simultaneous detection and quantitation of the gram negative pathogenic bacteria based on lipopolysaccharide (LPS) component was optimized. The exposure of the marine animal lysate blood to the polysaccharides of gram negative pathogens results in activation of an intracellular coagulation. The hypothesis is the rate of coagulation is associated to the LPS chain length. Carbohydrate assay and turbidity assay are basis of the main methods used in this study. *E. coli*, *Samonella.* spp., and *V. cholerae* with similar initial cell count per ml had different absorbance readings by using the spectrophotometer and turbidity meter. The range of difference in absorbance is consistent between different selected gram negative genera. Two selected corresponding genes encoding the LPS are explored for future application as molecular detection assay. The *wzm* and *wzt* genes encoding O-polysaccharide genes were amplified in these pathogens and the LPS factor C were amplified from the marine lysate. The hybridization results clearly demonstrated that *wzm* gene, hybridized with anti-LPS factor C peptide of marine lysate, thereby; homogeneity of bacterial O-polysaccharide gene to factor C peptide could be exploited in genera differentiation method.

The significant impact of the study is the considerable advancement in the molecular protocols, molecular characterizations of local *E. coli* strains since protocols are highly optimized to suit local strains, and diverse *E. coli* sources were included in the study.

The present study established the framework for the potential improvement in methods using genotypic based approaches for sources tracing and related genes differentiation for identification and quantitation. The genomic based probe from the RAPD fingerprinting is notably worthy for *E.coli* species specific detection without PCR

approach and the newly developed epidemiological marker for *E. coli* from different sources is valuable in future molecular diagnostics of *E. coli*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENAMBAHBAIKAN KAEDAH MOLECULAR UNTUKPENGESANAN *ESCHERICHIA COLI PATOGENIK*

Oleh

NAGI AHMED ABDULLAH AL-HAJ

Mei 2007

Pengerusi : Profesor Madya Mariana Nor Shamsudin, PhD

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Cirit-birit merupakan penyebab utama berlakunya penyakit dan kematian di kalangan kanak-kanak negara membangun, di mana sebanyak 1.3 billion episod dan 4 hingga 10 juta kematian dianggarkan berlaku setiap tahun dalam kanak-kanak di bawah umur 5 tahun. Patogen penyebab cirit-birit yang biasa adalah 'Diarrheagenic *Escherichia coli*' (DEC), rotavirus kumpulan A, spesis *Shigella*, spesis *Salmonella*, *Campylobacter*, dan *Vibrio cholerae*. Pandangan dari sudut mikrobiologi berdasarkan ciri-ciri fenotip dan genotip adalah cara pendekatan yang bernilai dalam mengurus dan menghindar daripada tersebarnya wabak cirit-birit yang berpunca daripada *E.coli*.

Dalam kajian ini, kaedah Randomly Amplified Polymorphic DNA (RAPD) digunakan untuk penilaian kepelbagaian genetik ke atas 25 isolat *E.coli* menggunakan 20 primer berbeza. Enam daripada 20 primer khususnya OPAE 4,9,10,11,12 dan 18 menghasilkan corak cap jari DNA dengan kuasa pembezaan yang menunjukkan potensi penanda ini

corak cap jari DNA dengan kuasa pembezaan yang menunjukkan potensi penanda ini sebagai penanda diagnostik dan epidemiologi. Penemuan yang sangat penting daripada corak jari DNA ini adalah pada saiz 308 bp yang mana jalur-jalur tebal dan terang hadir apabila primer OPAE-10 digunakan. Jalur-jalur tebal dan terang ini sentiasa hadir dalam semua 5 strain *E.coli* dari pelbagai sumber, namun tidah hadir dalam isolat Gram positif atau strain kawalan Gram negatif yang lain.

Identiti jalur ini selepas analisis BLAST dengan TOPO 2.1 ialah jujukan klon protein *secD.* Penemuan yang berharga daripada aplikasi genotip ini adalah saiz yang kecil di mana 100% homologi kepada membran pengangkut protein *E.coli* iaitu protein *secD.* Sebagai tambahan, peratus yang tinggi iaitu 100% persamaan dalam RAPD menggunakan primer OPAE-10 kepada genom *E.coli* menunjukkan bahawa penanda ini mampu digunakan sebagai penanda spesifik spesies. Di samping itu, esei membran yang dihasilkan menggunakan prob yang dicipta berdasarkan jujukan penanda menunjukkan signal positif untuk semua strain *E.coli* tetapi tidak pada isolat Gram positif dan Gram negatif yang lain, di mana ini membuktikan bahawa penanda *secD* adalah primer dan prob yang spesifik untuk mengesan spesies isolat *E.coli.* Walaupun primer RAPD OPAE-10 boleh didapati secara komersil, primer RAPD OPAE-10 sebagai penanda diagnostik *E.coli* masih belum dilaporkan dalam menjeniskan *E.coli* dari mana-mana sumber diagnostik. Sistem mengkelas RAPD yang digunakan dalam kajian ini merupakan yang pertama yang menyertakan 5 sumber berbeza serentak.

Penilaian genetik yang lain dalam mengesan strain *E.coli* dari pelbagai sumber adalah berdasarkan gen *GAD.* Ini menjadikan gen ini sebagai calon gen lain bagi kajian penghasilan prob gen. Gen pada saiz 671bp ini dilaporkan hadir secara homogenus pada

kebanyakan strain *E.coli* dari pelbagai sumber antaranya sumber klinikal, haiwan, air laut, sungai dan makanan. Kesemua isolat *E.coli* yang diperiksa membawa gen GAD pada 671bp. Esei membran menggunakan teknik hibridasi dot-blot mengaplikasikan prob oligonukleotida yang dicipta dari jujukan gen *GAD* bukan sahaja sangat sensitif dan spesifik ke atas semua isolat *E.coli*, malah ia merupakan esei yang cepat yang dihasilkan supaya dapat mengesan dalam masa yang singkat.

Penilaian kajian genetik ke atas strain *E.coli* membolehkan teknik berasaskan PCR dihasilkan dalam penentuan strain pembawa penyakit. Sumbangan enam pasang primer khas untuk gen-gen *stx1* dan *stx2* dari 'enterohemorrhagic *E.coli*' (EHEC), gen-gen *bfp* dan *eae* dari 'enteropathogenic *E.coli*' (EPEC), gen *elt* 'enterotoxigenic *E.coli*' (ETEC), dan gen *EAST* 'enteroaggregative *E.coli*' (EAEC) membantu dalam menghasilkan kaedah pengesanan pantas ke atas kumpulan *E.coli* dari pelbagai sumber dalam kaedah PCR tiub tunggal. Sebagai tambahan, kajian pengesanan berdasarkan gen virulen ke atas strain pembawa penyakit meggunakan Lipopolisakarida (LPS) yang didapati pada bakteria Gram negatif telah digunakan dalam menghasilkan kaedah genetik yang efektif.

Kaedah mudah berdasarkan hasil-pecahan biologi untuk pengesanan serentak bakteria patogen Gram negatif berdasarkan komponen lipopolisakarida telah diperbaiki. Pendedahan hasil-pecahan darah haiwan marin kepada polisakarida patogen Gram negatif mengaktifkan penggumpalan dalam sel. Hipotesis di sebalik kajian ini ialah kadar penggumpalan adalah berkaitan dengan panjang rantaian LPS. Esei-esei karbohidrat dan kekeruhan merupakan kaedah utama dalam kajian ini. *E.coli,*

Salmonella spp., dan *Vibrio cholerae* dengan kiraan awal sel per mL yang sama mempunyai bacaan resapan yang berbeza tetapi konsisten dengan menggunakan spektrofotometer dan meter kekeruhan. Ciri molekul LPS yang berkait dipelopori untuk kegunaan masa depan dalam esei pengesanan gen peringkat molekul. Gen *wzm* dan *wzt* yang mengkodkan gen-gen O-polisakarida digandakan dalam patogen-patogen ini dan faktor C LPS digandakan daripada hasil-pecahan marin. Keputusan hibridasi dengan jelas menunjukkan gen *wzm* berhibridasi dengan peptida anti-LPS gen faktor C hasil-pecahan bio marin, di mana homogenesiti gen bakteria O-polisakarida kepada peptida factor C boleh dieksploitasi dalam kaedah pembezaan genera. Kesan ketara dari kajian ini adalah kemajuan yang agak pesat dalam protokol peringkat molekul, pencirian molekul strain-strain *E.coli* tempatan memandangkan protokol diperbaiki bersesuaian dengan strain-strain tempatan, dan *E.coli* pelbagai sumber dimasukkan dalam kajian ini.

Diversiti genetik yang dipamerkan dari corak cap jari DNA adalah berdasarkan corak kerentanan antibiotik, Tujuh puluh isolat *E.coli* dari pelbagai sumber menunjukkan corak kerentanan yang pelbagai kepada 10 ejen antimikrob yang diuji, 61.2% daripada isolat *E.coli* diambil semula untuk pemprofilan kerentanan antibiotik. Secara keseluruhan, tetracycline dan kanamycin merupakan agen antibiotik yang paling lazim dilaporkan iaitu sebanyak 81%, diikuti dengan chloramphenicol 76%, gentamycin 72%, dan ampicillin 73%. Sementara itu, kerentanan yang paling rendah dapat dilihat terhadap ciprofluoxacin (24.0%), norofluoxacin (27.0%), dan cefutoxin (40.0%). Kaedah pengenalpastian biokimia konvensional berdasarkan fenotip dilakukan dalam kajian ini untuk mengesahkan identiti strain setiap bakteria yang digunakan dalam kajian ini.

Kesimpulannya, kajian ini telah mengukuhkan rangka kerja untuk memperbaiki potensi kaedah berasaskan genotip dalam mengesan sumber dan pembezaan gen berkaitan bagi tujuan pengenalpastian dan kuantitasi. Prob berasaskan genom cap jari RAPD ini adalah penemuan penting yang bernilai ke atas pengesanan khusus isolat *E.coli* tanpa pendekatan PCR.

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