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Polymerase chain reaction detection of haemolysin D gene (*hlyD*) in uropathogenic *Escherichia coli* as a novel diagnostic test for urinary tract infection

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Abstract:

Background: Urinary tract infection (UTI) is a common and sometime serious infectious disease diagnosed using conventional urine culture as the 'gold standard' for identifying *Escherichia coli,* the most common causative agent. However, due to the slow turn-around-time and other challenges of urine culture, this study explores the use of a novel biomolecular polymerase chain reaction (PCR) approach to detect the presence of haemolysin D gene (*hlyD*) that encodes a unique virulence factor of uropathogenic *E. coli* (UPEC) for its rapid identification in UTI.
Methodology: Primers from UPEC CFT073 and non-pathogenic *E. coli* K-12 MG1655 strains provided by Nottingham Trent University, England, UK were used to investigate the presence of haemolysin D gene (*hlyD*) in UPEC. The *hlyD* primers were developed from *hlyD* with locus number C_RS01660 on UPEC CFT073 strain using the NCBI, virulence finder, and Island viewer, and used in a PCR assay to target the *hlyD* in UPEC. Three sets of PCR templates were designed (UPEC, *E. coli,* and "No template"), each with internal and external controls amplified in a multiplex PCR assay, and agarose gel electrophoresis was used to separate the amplicons, and determine the specificity of *hlyD* for UPEC.
Results: The UPEC genome PCR assays were positive for *hlyD* and UPEC positive control, and similarly, PCR was positive for *E. coli* genome positive control, but negative for *hlyD*. Moreover, the "No template" PCR assay was clean with no amplification product, confirming the absence of PCR contaminations.
Conclusion: The *hlyD* is a unique virulence gene specific for UPEC. PCR assay of this gene is a promising specific and rapid biomolecular diagnostic test that can overcome the limitations of the traditional approaches for detection of UPEC in UTI.

Keywords: UTI, uropathogenic *Escherichia coli,* virulence factors, PCR, diagnosis

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Détection par réaction en chaîne de la polymérase du gène de l'hémolysine D (*hlyD*) dans *Escherichia coli* uropathogène comme nouveau test de diagnostic pour l'infection des voies urinaires

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Résumé:

Contexte: L'infection des voies urinaires (UTI) est une maladie infectieuse courante et parfois grave diagnostiquée à l'aide d'une culture d'urine conventionnelle comme «étalon-or» pour identifier *Escherichia coli*, l'agent causal le plus courant. Cependant, en raison du délai d'exécution lent et d'autres défis de la culture d'urine, cette étude explore l'utilisation d'une nouvelle approche biomoléculaire de réaction en chaîne par polymérase (PCR) pour détecter la présence du gène de l'hémolysine D (*hlyD*) qui code pour une virulence unique facteur d'*E. coli* uropathogène (UPEC) pour son identification rapide dans les infections urinaires.
Méthodologie: Des amorces provenant des souches UPEC CFT073 et non pathogènes d'*E. coli* K-12 MG1655 fournies par l'Université de Nottingham Trent, Angleterre, Royaume-Uni, ont été utilisées pour étudier la présence du gène de l'hémolysine D (*hlyD*) dans l'UPEC. Les amorces *hlyD* ont été développées à partir de *hlyD* avec le numéro de locus C_RS01660 sur la souche UPEC CFT073 à l'aide du NCBI, du détecteur de virulence et de la visionneuse d'île, et utilisées dans un test PCR pour cibler le *hlyD* dans UPEC. Trois ensembles de modèles de PCR ont été conçus (UPEC, *E. coli* et «aucun modèle»), chacun avec des contrôles internes et externes amplifiés dans un test de PCR multiplex, et une électrophorèse sur gel d'agarose a été utilisée pour séparer les amplicons et déterminer la spécificité de *hlyD* pour l'UPEC.
Résultats: Les tests PCR du génome UPEC étaient positifs pour *hlyD* et le contrôle positif UPEC, et de même, la PCR était positive pour le contrôle positif du génome *E. coli*, mais négative pour *hlyD*. De plus, le test PCR "sans matrice" était propre sans produit d'amplification, confirmant l'absence de contaminations par PCR.
Conclusion: Le *hlyD* est un gène de virulence unique spécifique de l'UPEC. Le test PCR de ce gène est un test de diagnostic biomoléculaire spécifique et rapide prometteur qui peut surmonter les limites des approches traditionnelles de détection de l'UPEC dans les infections urinaires.

Mots clés: IVU, *Escherichia coli* uropathogène, facteurs de virulence, PCR, diagnostic

Introduction:

Urinary tract infection (UTI) is a crucial health issue, affecting almost 175 million people globally (1). Particularly in women, UTI is the most frequent infectious disease often associated with severe outcomes (2). A spectrum of UTI infection spans from asymptomatic to symptomatic bacteriuria, and acute UTI to chronic forms that can lead to serious conditions such as pyelonephritis and cystitis (1). Severe UTI is usually recurrent, difficult-to-treat, and may be fatal (3). Approximately $3 billion is spent each year on UTI management in the United States healthcare system (1).

Uropathogenic *Escherichia coli* (UPEC) remains the main etiologic agent of uncomplicated UTI accounting for at least 80% incidence (2,4). A similar statistic was reported in the US regarding community-acquired UTI, and UPEC usually coexist with *Staphylococcus saprophyticus*, a Gram-positive agent of UTI with a prevalence of 10-15%, and often have a similar presentation among humans, with associated bacteriuria (1). Rapid identification of UPEC is crucial for patient management and quick epidemiological investigations.

Escherichia coli, a member of the family *Enterobacteriaceae*, is a gastrointestinal tract symbiont, but many pathogenic *E. coli* strains with varying virulence genes can cause severe infections (5,6). UPEC is a common extraintestinal pathogenic *E. coli* (ExPEC) that causes community-acquired UTI associated with morbidity and mortality globally due to the large range of associated virulence genes (5,7). The ExPEC is also known for its carriage of many virulence genes in pathogenicity islands (5) such as toxins (*hlyA, cnf*, and auto-transporter), iron acquisition and transport systems, adhesins, invasins, lipopolysaccharides, proteins, and siderophores. These factors equipped the pathogen with the ability to colonize and invade the urinary tract through the blood stream, and also empower ExPEC with antibiotic resistance (3,8). The UPEC virulence factors are two-fold; secreted toxins and factors found on the bacterial cell surface (5). Secreted *E. coli* toxins facilitate illness through inflammation activation (5).

Haemolysin D (*hlyD*) is an essential constituent of typical alpha-haemolysin (*hlyA*) along with *hlyB* and *tolC*, forming a transporter complex through which *hlyA* can be directly moved to the extracellular medium (5). Furthermore, haemolysin production confers *E. coli* with higher pathogenicity and persistence in the host, leading to recurrence of extra-intestinal infections (9,10). Exploring the relevant virulence/pathogenic genes versus the strain type is of great importance for diagnosis, therapy, and vaccine development for pathogenic *E. coli* (11).

Laboratory approaches for the diagnosis UTI include urine dipstick, culture, and microscopic urinalysis (12). At present, the standard urine culture is the 'gold standard' test, being highly reliable (95% sensitivity and 85% specificity), and although it is able to differentiate *E. coli* from other uropathogens, the turnaround time is prolonged, making it unsuitable for prompt therapy of uncomplicated UTI. Other setbacks of urine cultures include failure to grow atypical microbes and false-positive culture results from contamination (13). Rapid UTI kits have a high false-positive rate, inaccuracies, and low specificity, even though more promising modalities are being investigated (14). Contrastingly, the flow-cytometer-based urine analyzer can scr-

een out two-thirds of samples that did not require standard urine culture, thereby decreasing the urine culture workload (15).

The limitations encountered in the traditional biochemical testing methods of *E. coli* give way to molecular diagnostic techniques such as the polymerase chain reaction (PCR) assay (2). PCR assay is now the most successfully recognized technique for the detection of nucleic acids (2), targeting different genes of specific uropathogens (1). It is used for rapid identification of specific bacterial pathogens through the use of primers for several targeted genes in one reaction tube cutting down on turnaround time and costs (13). This study aims to determine the specificity of the *hlyD* virulence gene in UPEC as a diagnostic target in PCR assay.

Materials and method:

Study setting:

The study was conducted in 2017 as part of the Master of Science (MSc) course works of the corresponding author in Biomedical Science at the Microbiology Laboratory of Nottingham Trent University, England, United Kingdom

Bacteria strains and primer designs:

UPEC CFT073 and K-12 MG1655 strains (provided by Nottingham Trent University) were used in this study as PCR genome templates for UPEC (positive and internal controls) and *E. coli* primers respectively (Table 1). The extraction, purification and PCR assay of the bacterial DNA to obtain these PCR templates were done using GeneXpert system (Cepheid, USA), and made available to the main author by the module tutor (Dr Jonathan Thomas). These were used to investigate the presence of *hlyD* as a virulence gene in UPEC.

The unique *hlyD* virulence gene was first identified in UPEC as a diagnostic target using PCR assay, bioinformatic and comparative genome analyses. The *hlyD* with locus number (C_RS01660) on UPEC CFT073 and gene sequence (Table 1) was chosen and provided as a (UPEC primer) unique virulence gene, which was selected using the National Center for Biotechnology Information (NCBI), virulence finder, and Island viewer, in order to examine its presence in UPEC and *E. coli* by PCR assay in this study.

UPEC PCRs set up:

Master-mix was prepared in an Eppendorf tube to contain 35µl Mango Mix, 26µl water, and 3µl of UPEC CFT073 gDNA; 17µl of this master-mix was pipetted into each of the three labelled PCR tubes (UPEC pTest, UPEC pUPEC, and UPEC pEc) and then 1.5µl of each 10µM primer (left and right primers, control UPEC-F and control UPEC-R, and control Ec-F and control Ec-R) were also added respectively.

Non-pathogenic *E. coli* PCRs set up:

Master-mix was prepared in an Eppendorf tube to contain 35µl Mango Mix, 26 µl of water, and 3µl of non-pathogenic *E. coli* MG 1655 gDNA; 17µl of this master mix was pipetted into each of the three labeled PCR tubes (Ec pTest, Ec pUPEC, and Ec pEc), and then 1.5µl of each 10µM primer (left and right primers, control UPEC-F and control UPEC-R, and control Ec-F and control Ec-R) were also added respectively.

No template control PCRs set up:

"No template" control PCR master mix was prepared in an Eppendorf tube to contain 35µl Mango Mix and 26µl water; 17µl of this master-mix was pipetted into each of the three labeled PCR tubes (NTC pTest, NTC pUPEC, and NTC pEc), and then 1.5µl of each 10µM primer (left and right primers, control UPEC-F and control UPEC-R, and control Ec-F and control Ec-R) were also added respectively.

PCR amplification:

The amplification of the virulence gene was done in a PCR machine (Eppendorf Master Cycler). The nine labeled PCR reaction tubes (3 from each UPEC, non-pathogenic *E. coli*, and "No template" control) were placed in the PCR machine and ran under the following conditions; 1 cycle of 95°C for 90 secs, 35 cycles of 95°C for 30 secs, 52°C for 60 secs, 72°C for 90 secs, and 1 cycle of 72°C for 5 min.

Agarose gel electrophoresis of PCR amplicons:

Electrophoresis of the PCR products was done by pipetting 10µl of each of the nine prepared PCR reactions into the corresponding labelled wells of the agarose gel alongside 5µl of 1kb DNA ladder used as a molecular size marker. The agarose gel was placed in an electrophoretic machine and ran for 45 minutes under an electrical current at 120v (16,17). The DNA amplicons were visualized in the gel under ultraviolet transilluminator as bands following ethidium bromide staining (18).

Results:

Fig 1 shows gel electrophoresis picture of the PCR amplicons of *hlyD* primers in UPEC CFT-073 and *E. coli* K-12MG1655 strains. For UPEC genomic PCR, the result is *hlyD* positive as the test result in lane 1 is positive with a size amplicon of ≥ 300 base pairs (bp). Also, *E. coli* control (K-12 MG1655) PCR in lane 2 is positive with a big size amplicon of almost more than 700 bp, and UPEC control (CFT073) PCR in lane 3 was very weak with primer-

dimer of less than 300 bp (weakly positive). For *E. coli* genomic PCR, the result is *hlyD* negative as the test result in lane 4 appears with no DNA band, and the UPEC control (CFT073) PCR in lane 6 is also negative with no products, only the *E. coli* control (K-12 MG 1655) PCR in lane 5 showed positive DNA band of almost more than 700 bp. For the "No template" PCR, the lanes were clean with no products, indicating that the PCR preparations were good with no contamination.

Table 1: DNA sequences of primers designed for the study

Genome/gene	Forward sequence/Reverse sequence (F/R)	Length	T_m (°C)	GC%
UPEC (CFT073)	F- 5'-GTGGCAGTATGAGTAATGACCGTTA-3'	25	56	44.0
	R- 5'-ATATCCTTTCTGCAGGGATGCAATA-3'	25	54	40.0
E. coli (K-12 MG1655)	F- 5'-ATGGAAAGTAAAGTAGTTGTTCCGGCACA-3'	29	59	41.4
	R- 5'-GGACGCAGCAGGATCTGTT-3'	19	53	57.9
Haemolysin D (*hly D*)	F 5'-GGTTCAGCGAGTTCCTGTTG-3'	20	54	55.0
	R 5'-TACCGAGTTTGTTCCAGCCT-3'	20	52	50.0

T_m = annealing temperature; GC = Guanine-cytosine content; UPEC = uropathogenic *Escherichia coli*

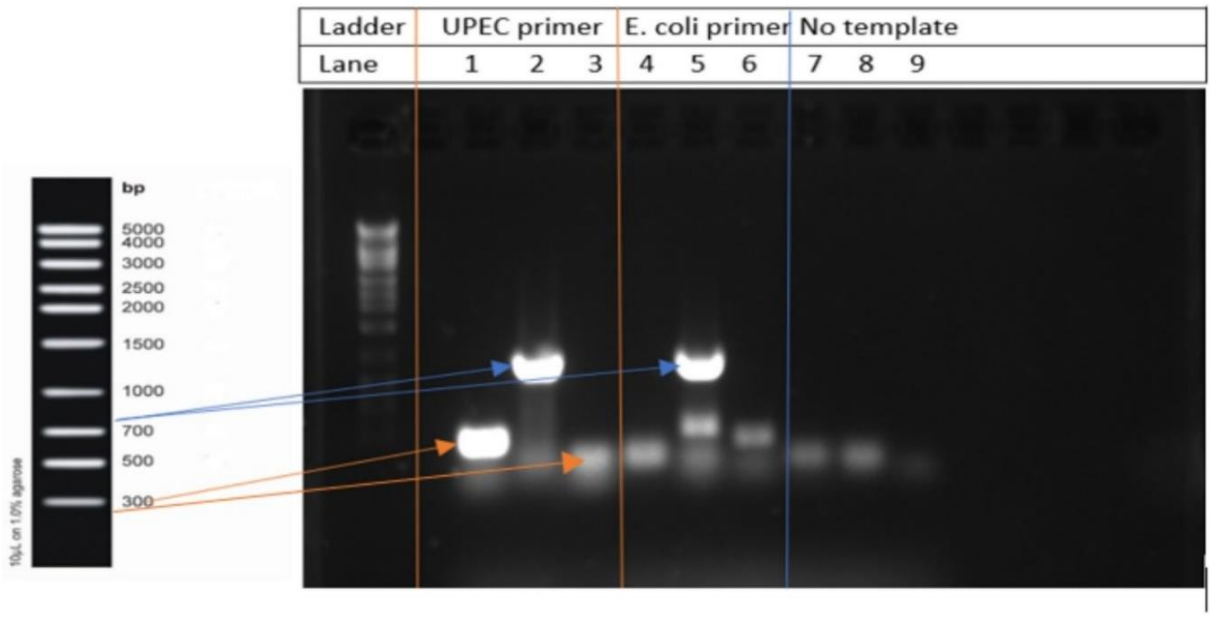

Fig 1: Gel electrophoresis of the PCR products observed through ultraviolet transillumination

The first column is a molecular ladder lane, the second column are UPEC genomic amplicons, the third column are *E. coli* genomic amplicons, and the fourth are "No template" amplicons. Lane 1 is UPEC test (*hlyD*) positive amplicon, lane 2 is *E. coli* control amplicon, lane 3 is UPEC weak positive control amplicon (with primer-dimer), lane 4 is *E. coli* test (*hlyD*) negative amplicon, lane 5 is *E. coli* control amplicon, lane 6 is UPEC negative control amplicon, and lanes 7, 8, and 9, are "No template" negative amplicons.

Discussion:

Simple urinary tract infection is a common condition, almost 50% of women globally will have an incidence of at least an episode of UTI in their lifetime. As a frequent health challenge, it can result in reduced productivity, poor ill health, and enormous economic costs each year (4). The haemolysin D virulence factor is of particular importance in UPEC colonization of the urinary tract and induction of major inflammatory pathways such as NF-κβ through TLR-4 signaling (5). Rapid identification of UPEC in UTI is therefore crucial for patient management and quick epidemiological investigations.

In this study, the designed *hlyD* primers detected only UPEC and not *E. coli* genomes, which implies that primers for *hlyD* are specific only for UPEC. In addition, for a valid result, the UPEC positive control primers should only detect UPEC and not *E. coli* genomes. In this study, UPEC positive control primers detected only UPEC genomes though with a very weak expected amplicon size of nearly 300 bp, but negative in the case of *E. coli* with no detectable amplicon product. This validated that the PCR conditions were optimal (primers, UPEC genomic DNA, and deoxynucleotide triphosphates). However, in the case of *E. coli* control primers, the primers can detect any type of *E. coli* genomes. Therefore, in this study, the *E. coli* control primers detected both the UPEC genome (lane 2) and the *E. coli* genome (lane 5) all with greater than or equal to 700bp amplicon size. There was no amplicon in the template control PCRs in this study, indicating the absence of contamination in both the PCR preparations and reactions (19).

The notable finding of this study is the fact that PCR could differentiate UPEC from non-pathogenic *E. coli* by detecting the specific *hlyD* virulence gene. Vysakh and colleagues (20) reported similar findings in their multiplex PCR assays using *hlyD*, *papC*, and *sfa* genes to target biofilm-forming rapidly growing UPEC (strain BRL-17) in a urine sample of patients with severe UTI.

Conclusion:

Diagnostic PCR technique performed in this study was simple and specific for the detection of *hlyD* in UPEC strain. Accordingly, this approach can serve as alternative to the traditional urine culture for the detection UPEC in UTI. However, there is the need for further investigations of this assay directly on urine samples to confirm this result.

Contributions of authors:

ANH conceptualize the study and performed the laboratory analysis; ANH, AM, and JM perform literature search and wrote the initial draft; ANH, MY, JM, and AM wrote the final draft; AM, JM and MY performed critical revision of the manuscript; and ANH and AM revised the manuscript. All authors agreed to the final manuscript submitted.

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Conflict of interest:

No conflict of interest is declared

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