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Comparative genomics of bacterial pathogens to design and validate a diagnostic PCR assay for detection of UPEC (UTI89)

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ABSTRACT

Urinary tract infections (UTIs) present clinical problems which can be caused infection by billions of organisms, including *Escherichia coli* as the main common cause. This invasion triggers a series of risk factors such as kidney failure or patient death in a worst-case scenario. Infection occurs when bacteria enter the bladder by escaping at micturition or as a result of contamination; these bacteria will directly colonize the bladder. UTI are most commonly found in women, which is basically related to the anatomical features of the female urethra. This paper concentrates more on the main methods of detection of UTIs. The entiregenome was determined and sequenced using bioinformatics analysis, which includes the programs Virulence Gene, Island Viewer and, most importantly, Blast N. Comparisons were made among all three strains of *Escherichia coli* to indicate the target gene, which was subsequently used as a PCR primer target.

Keywords: Urinary tract infections, *Escherichia coli,* PCR primer target

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INTRODUCTION

Uropathogenic *Escherichia coli* (UPEC) are the major cause of urinary tract infections (UTIs), being responsible for around 80-90% [1-6]. *E.coli* is a nonsporing gram-negative bacillus around 0.6-1.0 mm wide and 2-3mm long. It was discovered by the Germany bacteriologist Theodor Escherich in 1885, and hence the name. It was classified under the Enterobacteriacae group [7- 8]. This type of bacteria, with an overall rod shape, can be found in the intestines, normally in the human gut, and indeed can be very important for the body [5, 9, 10]. Most bacteria of this kind are entirely harmless; however, some of them are stereotype in nature and may threaten human life [1-3]. What is effective in helping these strains achieve pathogenesis is their typical gram-negative cell wall with an outer hydrophobic membrane which contains lipopolysaccharides (LPSs).Also effective is their ability to produce specific virulence factors; they include a block of DNA that causes this pathogenicity, and consequently produce these factors. They have peritrichous flagella and possesses fimbria, which are very important in adhesion and are considered to be the crucial virulence factors [1,2,3,9] in *E.coli*, which is related to a variety of diseases such as diarrhoea and dysentery in the gastrointestinal tract, as well as kidney failure and bladder infections, to meningitis and septicaemia. The reasons for the virulence of *E.coli* are related to habitat and genetic causes. This means that changes in virulence factors can easily lead to a variety of diseases, and thus why this type of bacteria is the underlying cause of various diseases, symptoms and causes. [2] There are different strains of the *E.coli* virotype which comprises uropathogenic *E.coli* (UPEC) strains. This type is considered the principal cause of UTIs because it produces haemolysins, which are responsible for lysis in the host cells. One of the most fundamental features of this bacterium is its capacity to colonise the bladder after becoming prevalent in the intestinal tract. Generally, the UPEC can provide type I pili, which contacts easily with mannose and contains glycol proteins, by using protein adhesion. Fim H will locate on the bladder surface. Basically, when the bacterium comes into contact with the superfacial cells, it will proliferate and enter the bladder. To illustrate this mechanism, the bacteria will change the virulence protein which frequently results in associated changes to the pathogenicity [1, 2, 3, 6, 7, 8] (Figure1).

MATERIALS AND METHODS

The method was divided into two stages. Firstly, bioinformatics workshops, which constituted three different stages by using bioinformatics, design. The second stage of this study was carried out by diagnostic PCR-based targets in order to compare the genomics of the bacterial pathogenesis.

Bioinformatics search

In this set of bioinformatics workshops, this study attempted to determine the presence of any unique genes in uropathogenic *E. coli*, which can be used as diagnostic PCR targets [18]. Bioinformatics is basically the application of information technology to advance biological research [12-14]. It is a crucial technique to obtaining fast and convenient results. The first IT work covered how to distinguish between Fasta files and Gen bank files. Fasta files give us the nucleic acid sequences for the genes we selected but did not otherwise contain much information, while Gen bank provides a great deal of information including the amino acid sequence for every gene and genome sequences that refer to Gen bank contain all the information and the function for the selective genome. To detect the genome of interest, accessed the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov\genome\). Which is a repository for massive databases that make it easy to locate specific genomes. After searching for *E.coli* strains on this site, we were looking for specific genome pages to select (reference genomes). We chose non-pathogenic intestinal strains such as MG1655 and K12 as the reference genomes. Among the three database selections (Graphics, Fasta and Gen bank) we choose Fasta files to gain specific genomes and downloaded the sequences in fasta format, which were then saved on computer under the name of the relevant bacteria. These were then opened in ARTEMIS (www.sanger.ac.uk\software\ACT), which is a popular visualisation program for genome comparison. The same steps were followed for the other strains and for Gen bank files, but the different was using customize then view. These steps were completed for *E.coli* K12 strains and later for UTI89 and SakaiO157 as well. Using the same steps as mentioned previously, to gain a clear understanding of these strains you need to identify the most important differences among them, noting in particular that UTI89 is an uropathogenic (UPEC) *E.coli* strain, while SakaiO157 is a diarrheic enterohemorrhagic strain which results in blood in the stool and that also releases a toxin. MG1655, by contrast, is a harmless and non-pathogenic strain. After detecting the three strains of *E.coli*, the genomes were submitted to the virulence finder website (http://cge.cbs.dtu.dk/services/VirulenceFind/) for comparison. At the same time the submission to Fasta file was performed to detect the virulence genes. Certainly, this program provides us with the virulence genes for all three strains because it contains comprehensive information about all types

of virulence factors for the gene. This made it possible to compare between the specific genome of *E.coli* and the database to identify virulence genes in our list of bacteria. In the second IT work the virulence genesforMG1655, SAKIAO175 and UTI89 were identified by detecting different regions of pathogenicity island (PI) online (www.pathogenomics.sfu.ca/islandviewer/query.php). UTI89 was selected from the list and submitted to the Island Viewer webpage, and the pathogenicity island for UTI89 determined. The same steps were repeated for the other two strains. Simple observation then allowed for a comparison of Pathogenicity Islands of the three strains of *E.coli* and examination the gene in the pathogenicity island. By randomly clicking on any of this strain, for example, will display the virulence genes that allow one to find any relevant pathogenicity islands for all types of *E.coli*. The initial review of the results for all the strains showed there was a significant difference between their pathogenicity islands, which suggests that those genes in UTI (PI) might be unique to UPEC. For the next stage we used this website: https://lfz.corefacility.ca/Panseq. Click on the Novel region finder in order to detect the region of gene sequence. This displays the complete genome. We selected MG1655 and SAKIAO157 from the reference list and UTI89 from the query list. By clicking on "analyse", we found the regions of the genes which are unique to UTI89. In order to be sure about the results in pan genome analysis, UTI89 was selected in the query column and all the other non-pathogenic in the reference list. In the last IT work, we focussed on detecting some of UTI89's virulence genes via the pan sequence. It was estimated in this session that the virulence genes are unique toUTI89UPEC. Gen Bank file was opened in the ARTEMIS program, after which we performed a new program BLAST on NCBI webpage. BLAST is a very intuitive program, and was used to help identify UTI89 sequence nucleotides. By searching for all the genes in BLAST, it was possible to investigate whether they were unique for UTI89 or otherwise. Also, answering the question if it is uropathogenic E.coli or not was able to be addressed. In fact, there are fundamental factors which can help us to identify a unique gene. Firstly, we need to select the right genome for the comparison. Secondly, we need to apply virulence genes or other information and PI plasmids as well. Finally, in our workshops in the ITprogram, the most relevant methods were considering percentage similarities, such as 100% and 99% with *E.coli*, or by searching for a high similarity to UPEC. This indicates that a given gene is specific to UPEC and can be used as a PCR target for *E.coli* (UPEC) (Figure2).

PCR primer design: Polymerase chain reaction (referred to above, and herein, as PCR) is a technique which can provide crucial results for a variety of biology experiments, such as in molecular biology. PCR needs ideal conditions in order to produce good results and products [16-17]. This application is extremely powerful and can provide us with highly accurate solutions on only a short timescale. Furthermore, it is becoming cheaper, enormously sensitive, and quite specific, thus giving a more accurate result in less time. In this section, the target was to set up PCRs using primers designed. First of all, IT work were intended to help select the ideal primer, which was achieved by identifying the most essential features of good primers. Firstly, two primers rather than one were necessary, one for each strand, because the first, the forward primer, should bind with the strand in the 5' to3' direction; the second, the reverse primer, should be complementary in the 3' to5' direction. In order to help us to identify the starting point and the end, the sequence has been amplified [15,16]. Also, to design good PCR primers, we should consider the length of primer. Ideally, this should be around 500 bp, and the nucleotides bases between 15-30bp. Furthermore, the GC content should be in the range of 40-60% because the connection between the G and C is stronger than A and T, so we need to provide at least two GCs at the end of our primer (GC clump) because, if not, the primer will change place and then it becomes difficult to bind and amplify at the end [17,18]. Moreover, the ideal melting temperature (TM) for primers should be around 52-58^{o}C, though it can be 45-65^{o}C [19, 20]. In addition, it is important to avoid repeats in the nucleotide bases, for example, AAAA or GCGCGC, because this will lead to preform hairpin and slipping in the primers. Also, the DG must be more than -3 for hairpin and more than -5 for dimers. After the perfect primer has been identified, the next step is to design our primer from our unique gene (CNF1) that we selected during our bioinformatics analysis, which can be achieved through the www.idtdna_com\analyzer website. By clicking on "sequence DNA" then "Analyse", it is possible to check the most important characteristics such as hairpin and self-dimer formation in the primers. The essential ideal primer was selected to identify the sequence for the specific gene that we obtained during the bioinformatics research, which was then ordered. One gene (CNF1) was selected, as we believed it was specific to UPEC for the reasons given previously. Later, the Gen Bank file was opened, selection" then the "Fasta format", the sequence of our unique gene. The next step was to select the forward primer and reverse primer for the gene. This was completed by choosing the forward as being from 5' to 3' while the reverse was from 3'

to 5'. To gain on the reverse complementary we used this website: http://www.bioinformatics.org\sms\rev com.html. We determined the ideal characteristics as explained above, and then selected our new primer depending on these conditions. We then used this website:http://www.idtdn1.com\analyzer\aplicants\ oligoanalyzer\. Our focus was to check the TM, length, GC content, dimer and hairpin. In the last step in our IT session, the primer was selected then we put it in the field to analyse it (Analyser website) or used another website (Oligocalc) to detect our new primer. The primer that we requested for the gene (CNF1) was expected to be ready by the following workshop.

The aim of this study was to set up PCRs using primers designed to differentiate between UPEC and non-pathogenic *Escherichia coli*. To apply this stage we needed to provide the following material: PCR tube rack, Eppendorf p2 and p10 pipettes, tips and Mango PCR mix (which includes DNA polymerase, dNTPs, buffer and the gel loading dye), PCR primer, PCR-grade water, and the most important material is DNA genomic which has been isolated from the UPEC and non-pathogenic E.coli. The strains that we need to detect are FT073 (UPEC) and MG1655 (non-pathogenic *E. coli*

RESULTS

Bioinformatics results: During stage 1 of our study in the bioinformatics workshops, that is, when we compared the virulence genes of the UTI89, MG1655 and sakia0157 genomes, some genes were found in all three strains of *E.coli* (Table 1). UTI89 has some unique genes which are not found in the other strains, as does Sakia; however, MG1655 does not have any unique genes. The virulence finder results showed thatMG1655 non-pathogenic has just four virulence genes, while Sakia0157 contains 18 and UTI89 eight virulence genes. This means that in our search via Virulence Finder, we found that UTI89 has eight genes but in fact not all of them are unique to UPEC because we also found them in other strains. While some of these genes, such as cnf1, vat, iron and sfas, are very specific to UTI89, we think that these genes are UPEC-specific virulence genes because they were not seen in other strains (Table 2).

During our study, we also observed the pathogenicity results showed the variety of regions which are unique to UTI89, which was helpful in detecting novel regions. Additionally, it was gained on different virulence genes and pathogenicity island for all our *E.coli* strains. The result was visualised in ARTEMIS and then a number of genes were selected to detect which of them were unique in a limited range. After choosing every single range to detect the sequences and select the virulence genes, it became apparent that some of these virulence genes had features such as fimbria and pili which can be used for adhesion, as mentioned previously.

For example, the gene papg was shown to use pillus adhesion; the same result as hek, while the gene cnf1 shows cytotoxic ext (Table 3). Compared with the PI for UTI89, the MG1655 genes appeared to be toxic and adhesive, while UTI89 genes were more fimbrial and haemolytic. In this finding we can state that this pathogenicity island is specific toUTI89 because it was different from the other strain's pathogenicity island. Furthermore, the pan sequence online results were used to determine whether these genes are unique to UTI89 through the gene production and function, from which we chose which of them might make appropriate PCR targets for UPEC.

Further results have shown that the last detection of the genes by using the more powerful program (BlastN). The reason behind our belief is these genes have a high identity with and similarity to *E.coli* strains. By looking at the bacteria which presented in the BlastN result list, the majority of these strains cause diarrhoea and might be found in the intestines. In this case, we avoided these genes and searched for the specific genes which shared the same features as UPEC in production and function. Table 4 reports some of the genes which we decided were unique to uropathogenic *E.coli*.

PCR Results: The sequence to our primer was detected using the Oligocalc website, where the gene CNF1 was shown to be unique for both primers after using BlastN. The forward primer was 5'GAGGTATCTGTTCCGCTTGG3', whose analytical features are as follows: length, 20 bp;GC, 40%; and TM, 56.7°C.The reverse primer was 5'CCAAGCGGAACAGATACCTC3' which had a length of 20bp, a GC of55% and aTM of53.8°C. The PCR application results are shown in Figure 3. The results illustrate that our primers could not detect the UPEC successfully. As can be seen, the DNA ladder is in the first line. The expected result shows that the UPEC primer test is in the second line, while the third line should detect only UPEC because it is a UPEC control template (Figure 4). Another expected result from our control PCR, which is shown in Figure5, is the ICD primers detect both non-pathogenic *E. coli* and UPEC in line 4. In line 6, there is no result because it is just template *E.coli*, while line 7 should detect the MG. primer *E.coli*. Finally, the last three tubes, (lines 8, 9, and 10) should not show any results because there was no template in any of these wells.

The negative result was obtained as a result of the lack experiences in preform PCR reactions, which led to an unexpected result. This may have occurred for various reasons, as explained during troubleshooting the PCR [20, 21]. It might be the DNA template was damaged during the work; this can be overcome by replacing the template with a new one. This will definitely have an effect on the sequence. A further problem is contamination, either when using the tube or, indeed, for a number of possible reasons, and which will lead to errors in the size of products. No production and this obviously what occurs in PCR reaction. This might be because of poor primer design, a problem with the length of the primer, or errors in the primer concentration. Also, missing ingredients in the reaction tubes will result in a loss of product. In addition to irrelevant conditions which might be used in the lab design and the primer concentration, either too low or mistaken, in this case the target PCR reaction will not be found in the DNA template. These are some of the issues which might lead to poor PCR primer design, which will certainly be controlled for in any future studies.

DISCUSSION

According to Hasman et al.(2013),UTI is a common inflammation in humans, particularly in women. There are high percentages of hospital clinical cases of UTIs, and cases within the community as well. For these reasons, UTIs require high sensitively methods of diagnosis. PCR represents just such a full diagnostic method, in spite of the high level of sensitivity which required [8-11]. Another study which is similar to our own isolated the HUC270 strain of *E. coli*, as conducted in2007; PCR was applied multiple and it was discovered that this strain has three virulence factors which formed the pathogenicity island. Most of these VFs include fimbriae adhesions. Furthermore, the study showed that *E.coli* can mix with different genes, as determined via the triplex PCR method. [27] In this finding, we note the similarities to our own results through the specific virulence genes that we gained in our bioinformatics analysis [21-23]. Karimian et al. (2012) demonstrated the first pathogenicity method to detect UPEC virulence factors in UTIs using PCR. The study used the same approach to detect the unique gene in *E.coli*. Interestingly, the same virulence genes were detected in the UTI89 strain, including cnf1, iroN and pap, with a high percentage of prevalence .This finding illustrates that UPEC, which include virulence factors in their genes such as fimH, may be the fundamental cause of UTIs in humans. This suggests that the present study successfully obtained unique genes, which we believe are specific to UPEC according to the virulence factors. [23-27].

According to a study conducted by Cusumano et al (2010), around 60% of women are affected by uropathogenic *E.coli* in the course of their lifetimes, as a result of their relatively short renal, anatomical shape and contamination from faecal matter[9], while in men it is less common and usually only occurs past50 years of age. Actually, the inflammation is acute and short lived but still has a strong risk factor. UTIs represent a huge clinical issue; around 85% are commonly acquired and 25% are acquired in hospital [10, 12, 21]. Why is *E.coli* an important issue that needs to be treated? A huge rate of recurrent UTIs are caused by the same type of bacterial strains, and even with antibiotic therapy recurrence is common. This infection can affect both the upper side of the urinary tract and the lower side. This paper attempts to demonstrate the detection of UPEC and the pathogenesis of UTI89 through comparison with other strains of non-pathogenic *E.coli* such as CFT073 (UPEC), MG1655 (K12), and EDL933 (EHEC). This study sheds light on modern methods such as bioinformatics analysis and providing PCR primer design to detect the genes unique to UPEC in order to avoid other methods' problems, such as the massive numbers of urine samples of clinical cases which might be needed for classical diagnostics, such as dip sticks or urine cultures *in vitro*. Although gaining urine samplesisnot a serious issue like other specimens and indeed is less difficult to work with, it still requires a fast tool to analyse the massive numbers of samples required in a hospital every day, as well as preformed genome sequences decreasing the diagnostic time from 1 to 2 days to isolation and around 12 hours for the sequencing analysis [9-10]. To summarize, *E.coli* a significant cause of severe infection as a result to its ability to produce virulence factors which include the production of toxins, adhesion and haemolysin, which need to a high level of technique in order to gain on faster and clearer diagnosis in ideally time [18-23].

Figure 1:

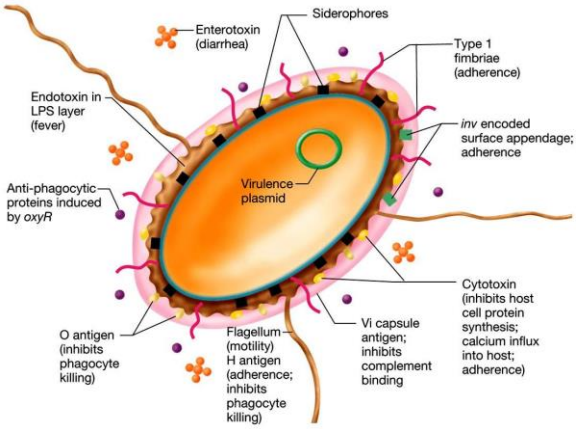


Figure 2. The method used to search forpan genome sequences.

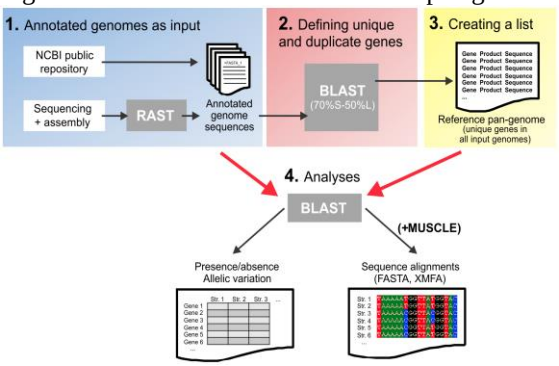


Table1.The virulence genes found in all the *E. coli*strains studied.

Uti89, MG1655, sakia0157
Iss
Gad
prfB

Table2. The unique genes detected in UTI89 and SAKIA0157.

SAKIA0157	**UTI89**
Tccp, strx2A	Sfas
Lha, nleA, astA	Iron
Gad, espj, stx1b	Vat
Nleb, espb, prfb	Cnf1
espa, Eae, tir, nlec	

Table 3. The virulence genes detected inthe UT189 pathogenicity island.

Gene name	**Production**
Papg	p pilus adhesion
papd	peri plasmid chaperon
papk	minor pilin subunit
Cnf1	cytotoxic necrotizing factor
hlyb	Haemolysin

Table 4 The list of genes unique to UPEC after running BLAST.

The virulence Gene name	production
Cnf1	Cytotoxic necrotizing factor 1
Papk	Adhesion, operon, and meningitis in high rates around 99% and 100%
Hlyd	Haemolysin D
Papj	Protein j
Iron	Outer membrane

Figure 3. The PCR reaction, showingthe negative results.

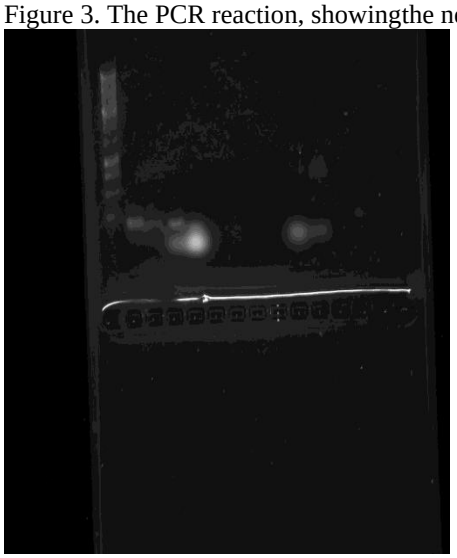


Figure 4. The expected result - PapC primers detect UPEC only.

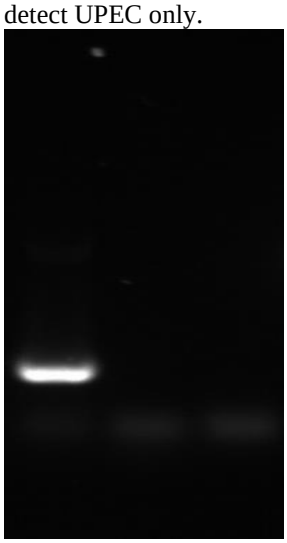
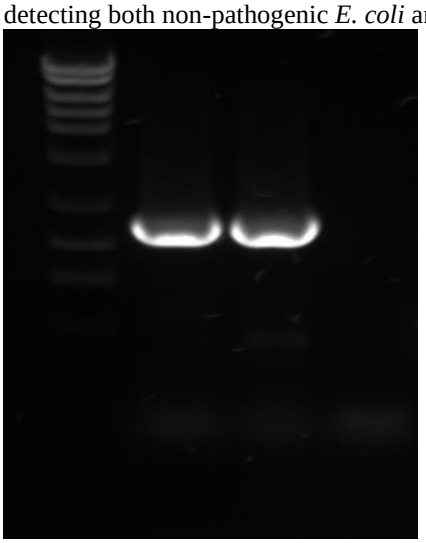


Figure 5.The expected result of ICD primers detecting both non-pathogenic *E. coli* and UPEC.



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