JOURNAL OF COMPLEMENTARY MEDICINE RESEARCH

Journal of Complementary Medicine Research, ISSN: 2146-8397 Vol. 13, No. 4, 2022 (pp. 163-170)

ORIGINAL RESEARCH ARTICLE

Genomic Analysis study of Virulence Potential α-hemolysin from Uropathogenic *E.coli*

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ABSTRACT

Uropathogenic *E.coli* (UPEC) is the source of numerous toxins that cause nosocomial and community-acquired UTIs, including -hemolysin, a secreted lipoprotein that is a powerful and ubiquitous cytolysin that belongs to the (RTX) family. Between March 2021 to December 2021, 123urine samples were collected from patients with urinary tract infections who were admitted Al-Hilla General Teaching Hospital in Al-Hilla City, from both sexes (male and female). Out of (123), 56(45.5%) were positive culture for *E. coli* which identified by selective media, biochemical tests, Vitek 2 system and *16s RNA* gene specific primer by the presence of (1492 bp) compared with allelic ladder, while other causes recorded 67(54.5%). DNA purification Kit supplemented by the manufacturing company Geneaid, (Korea). Primers were used and PCR condition to amplify sequences of the *16SrRNA* and *hlyA* genes. Amplification products were separated by electrophoreses. The gene sequencing for *16SrRNA* and *HlyA* gene was done by sanger sequencing method and phylogenic analysis. The study pointed to the number of mutation that happen with *HlyA* gene, as α-hemolysin gene varies greatly between local and international strains.

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How to cite this article: Al-Dulaimi TH, Bunyan IA, Banimuslem TA. Genomic Analysis study of Virulence Potential α-hemolysin from Uropathogenic *E.coli*. Journal of Complementary Medicine Research, Vol. 13, No. 4, 2022 (pp. 164-170).

INTRODUCTION

The prevalence of urinary tract infections, which are widespread, expensive diseases that impact millions of individuals, places a heavy load on the world's healthcare system. (Tewodros Tesfa *et al.*, 2021). Gram-negative and Gram-positive bacteria were the main cause of urinary tract infections. Numerous additional species, including *Escherichia coli, Proteus, Klebsiella, Staphylococcus epidermidis, Pseudomonas*, and others, were also identified as urinary tract infection causes in earlier investigations. (Al-Dulaimi, 2016). Many mammals' lower intestines and stools include the widespread bacteria *E. coli*. As a result, when it enters the urinary system, it multiplies, moves up to the urinary tract, and causes infection. This poses a serious public health issue in underdeveloped nations and is the primary cause (80-90%) of UTIs. (Terlizzi *et al.*, 2017). The ability of UPEC to bind host tissues is one of the key factors that facilitate UPEC colonization of the urinary tract, enabling the bacteria to withstand the heavy flow of urine and encouraging UPEC invasion of urothelial cells. UPEC preferentially colonizes the bladder and causes cystitis, but it can also ascend through the ureters into the kidneys, causing pyelonephritis. Numerous toxins found in UPEC, including -hemolysin, are linked to virulence mechanisms that are advantageous to bacteria. (Soltani *et al.*, 2018A type I secretion system (T1SS) that comprises an ATP-binding cassette transporter releases -hemolysin, a secreted lipoprotein that is powerful and widespread and belongs to the (RTX) family. It plays a significant role in pathogenesis and acts on a variety of distinct host cell types. (Vega *et al.*, 2019). This toxin produced by numerous pathogenic as well as commensal *Escherichia coli* isolates and has been proposed to function differentially over a gradient of concentrations. At higher toxin doses, the trans-membrane pores formed by *HlyA* can provoke colloid-osmotic (oncotic) lysis of eukaryotic cells including red blood cells (RBCs), epithelial cells and leukocytes, resulting in cell lysis while at low (sublytic) concentrations, hemolysin can either alter cell functions or provoke apoptotic cell death (Hongwe *et al.*, 2021). UPEC has strong or weak activity of hemolysin which effect on host cells at lytic and sublytic doses, so is particularly important and regulates multiple aspects of host cell biological processes, including the nuclear factor-KB (NFKB) signaling pathway (Rafiq *et al.*, 2018), induce apoptosis in epithelium cell of urinary tract and immunity cells, represent the classical and well-studied example of a pore-forming RTX toxin, induce the inflammation of the kidney and is an important virulence factor in pyelonephritis (Ristow and Welch, 2016), so is produced as either a free form or an outer membrane vesicle (OMV)-associated form. The free

KEYWORDS:

α-hemolysin, *16Sr RNA*, Phylogenetic tree. UPEC.

ARTICLE HISTORY:
Received : Jul 28, 2022
Accepted : Apr 00, 2022
Published: Jun 00, 2022

DOI:
10.5455/jcmr.2022.13.03.44

form irreversibly inserts into cell membranes of erythrocytes and epithelial cells, where depending on concentration it causes ion imbalance, structural changes, and cell lysis (Colleen *et al.*, 2021). The OMV-associated form is internalized by epithelial cells, where it targets mitochondria and triggers caspase-9-mediated apoptosis (Strack *et al.*, 2019). *HlyA* is induced kidney inflammation and a higher percentage of *hlyA*-positive strains are isolated from pyelonephritis patients (>70%) than from cystitis patients (31–48%), implying that *HlyA* is an important virulence factor in pyelonephritis (Ristow and Welch, 2016). An operon including the genes hlyC, hlyA, hlyB, and hlyD, which are positioned on the chromosome of pathogenic E. coli variations known as pathogenicity islands, contains the genes producing HlyA. The HlyA export, synthesis, and activation-related proteins are encoded by the operon. The genome of uropathogenic E. coli (UPEC) was molecularly analyzed, and it was discovered to have pathogenicity islands that are common among Escherichia coli species. (Radwa *et al.*, 2019).

AIM OF STUDY

The study was aimed to identification of UPEC isolates, and detect *hlyA* gene among UPEC isolates.

MATERIALS AND METHODS

Study Design

A total of (123) urine samples from patients were admitted to Al Hilla General teaching hospital (Urology unit), for the purpose to identification of UPEC isolates and genetic detection of hemolysin toxin among UPEC isolates during the period from March 2021 to December 2021.

Ethical Approval

All subjects involved in this work were informed and the agreement required for doing the experiments and publication of this work was obtained from each one prior the collection of samples. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee (at College of Medicine University of Babylon) under the reference No. BMS/0231/016.

Samples Collection Clinical Specimens

The samples collected from patients of different age groups arranged from (<10 - >52) years from patients suffering from UTIs, pyelonephritis and/or cystitis. Information on the gender, age, addresses collection and be surety that patient didn't take any medication for three days before collection of urine samples.

Clinical Specimens

About 10 ml of mid-stream urine samples from patients were diagnosed by a physician depending on clinical manifestation as patient with UTIs. The specimens were collected in the morning in sterile containers and be surety that patient didn't take any medication for three days before collection of urine samples. Urine specimens were inoculated on selective media at 37°C for 24-48 hours.

Diagnosis of Bacterial Isolates

The bacteriological study included all the urine samples were cultured on different media Blood agar, Eosin methylene blue and Chromo agar at 37°C for 24-48 hours. The characterization of isolated bacteria depended on microscopic features and biochemical tests (McFadden, 2000) and used an automatic identification (ID) instrument device Vitek 2 to validate clinical microbiology and biochemical tests.

Preparation of DNA: It was made according to the genomic DNA purification Kit supplemented by the manufacturing company Geneaid, (Korea). Chromosomal DNA obtained were used as templates for all PCR experiments

DNA Amplification: Primers were used and PCR condition to amplify sequences of the *16SrRNA* and *HlyA* genes were indicated in table 1. Amplification products were separated by electrophoreses on 10 mg/ml agarose gel (TopVision TM, Fermentas) in 1× TBE Buffer and ethidium bromide (5 µℓ) with a 1000-bp ladder (Fermentas) as molecular weight marker (Karimian *et al.*, 2012), The gene sequencing for *16SrRNA* and *HlyA* gene was done by sanger sequencing method and phylogenic analysis.

RESULT AND DISCUSSION

This result agrees with the findings of Hegazy et al., (2018) and Zahraa (2019), (53.85%) and (50%) respectively in their study of the isolation of bacterial causes of UTIs, and is higher than the (43.1%),(24.7%), recorded by Dhilal, (NCBI - National Center for Biotechnology Information), Ibrahim et al.,(2020), and respectively, and less than the (60%) recorded. The study revealed the dominance (2019).

Table 1: The primers sequences and PCR conditions optimal conditions for the polymerase chain reaction in UPEC

Gene name	*Primer sequence (5´- 3´)*	*Size of b.p*	*Conditions*	*References*
16S rRNA	F:AGAGTTTGATCCTGGCTCAG R: GGTTACCTTGTTACGACTT	1492	Step1:94ºC, 2min. Step2:98ºC, 10 sec. Step3:58ºC, 30sec. Step4:68ºC,1.5min. Step5: 68ºC, 5min.	(Lin *et al.*, 2008)
Hly A	F:AACAAGGTAAGCACTGTTCTGGCT R:ACCATATAAGCGGTCATTCCCGTCA	1177	Step1:94ºC, 3 min. Step2: 94ºC, 1min. Step3:61ºC, 30 sec. Step4:72ºC, 3min. Step5: 72ºC,7 min.	Yamamoto *et al.*, (2009).

Detection of hlyA gene:

The presence of (1177 bp) bands when compared to an allelic ladder allowed researchers to identify the presence of the hlyA gene in 21 (37.5%) of the uropathogenic Escherichia coli samples (NCBI - National Center for Biotechnology Information). These findings coincide with those made by Yazdanpour et al. in 2020, who discovered that 26% of E. coli isolates from urine contained the hlyA gene. According to Shahbazi et al. (2018) and Moeinizadeh and Shaheli (2018), the prevalence of the hlyA gene was detected in urine-derived E. coli isolates at a rate of 41.7%. (NCBI - National Center for Biotechnology Information) found that, *E. coli* isolates of have *hlyA* gene in rate (90.8%) and higher than the result of Huda, (2018) that found the ratio of the presence of this gene in UPEC was (16.6%) . The high number of virulence factors help the bacteria to survive and cause infection also increased the capability of the bacteria to travel from the anus, which is its natural habitat, to the urethral tube due to the short distance these two are from each other could be one of the most important reasons for UTIs (Raeispour and Ranjbar, 2018).

Identification of *E coli* done by using specific primer *16SrRNA* gene (1492bp) two of isolates were recorded in the gene bank with the accession number OL539543.1 and OL539542.1. *16Sr RNA* was used to ensure that all isolates *E. Coli* befor *HlyA* gene detection. The slow evolution rates of the former regions enable the design of universal primers that amplify genes across different taxa, whereas fast-evolving regions reflect differences between species and are useful for taxonomic classification (Shinichi *et al*., 2019).

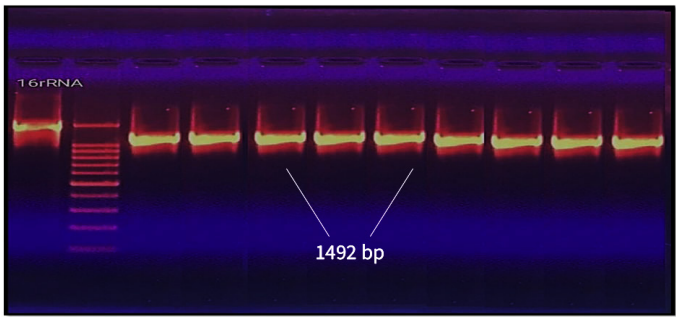

Fig. 1: Agarose gel electrophoresis at 70 volt for 50 min for *16srRNA* specific gene products visualized under U.V light at 301 nm after staining with ethidium bromide. Lanes were positive for *E. coli*; the size of product is (1492 bp)

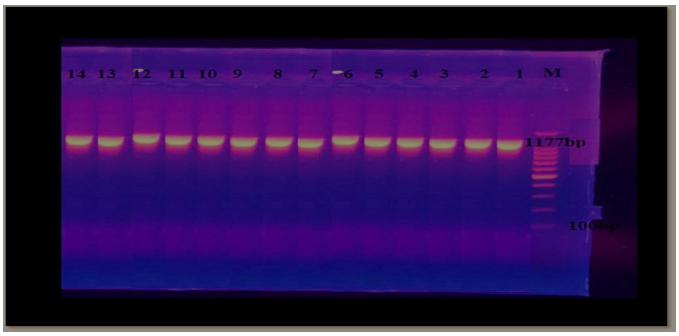

Fig. 2: *E. Coli* was identified via PCR detection of a 1177bp amplicon of the *HlyA* gene 100 bp DNA Lane M. Agarose gel electrophoresis of PCR products obtained by using hly-specific primer. lanes 1-14 represent the identified *HlyA*

PHYML was used to create a phylogenetic tree of Escherichia coli strains from the concatenated sequences of ten *HlyA* genes matching to 13 isolates of *HlyA* genes from NCBI. As it is known to be basal in the *E. coli* phylogeny, the tree has been rooted to five phylogroups. When the bootstrap value is more than 93 percent, it is reported at the nodes (500 replicates).

On the right of the picture, the main phylogenetic groups of *E. coli* species are displayed. The remaining strains were chosen as representative of *E. coli* phylogenetic diversity, as shown in the figure 3, with the Iraqi strain marked as a traingle, seven of the *HlyA* genes clustered together, and the other separated by other international strains, revealing that the effect of the hemolysin gene varies greatly between local and international strains. Therefore, our study pointed to the number of mutation that happen with *HlyA* gene which were in agreement with the research revealing a mutation in *rfaC* that altered both *Hly*A extracellular expression and activity was examined in greater depth.

This mutation resulted in a growth-phase-dependent decrease in the stable level for extracellular *HlyA* that really was up to 16-fold lower, despite the fact that transcription plus release of *HlyA* were only reduced by a factor of two. Specific hemolytic activity in the *rfaC* mutant strain toxin was dramatically reduced in a growth-phase-dependent way.

Both the reduced expression and activity of *HlyA* were recovered to wild-type levels when the *rfaC* gene was provided intrans. *HlyA* from the *rfaC* mutant strain had substantially slower hemolysis kinetics, a faster rate of activity decay, and higher production of apparently inactive *HlyA*-containing

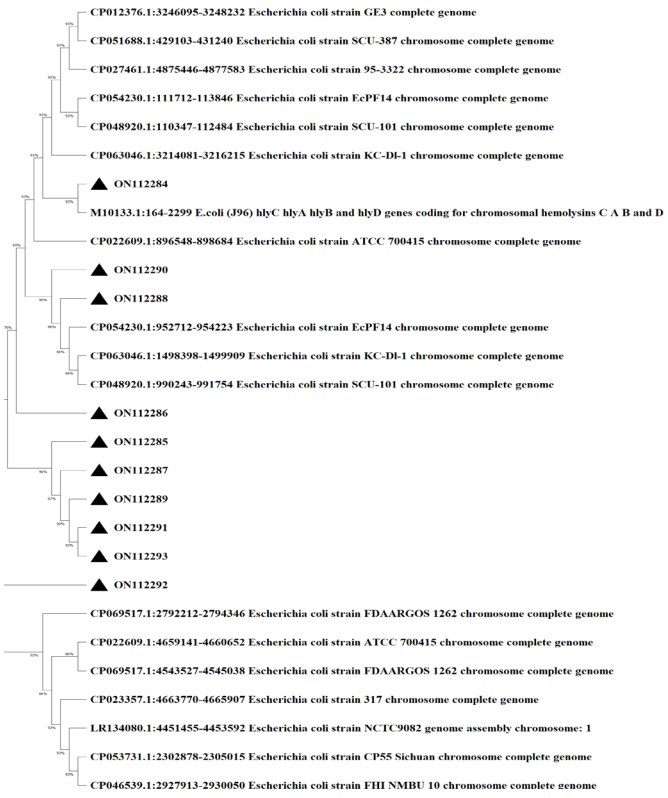

Fig. 3: Phylogenetic Tree for *HlyA* Gene of *Escherichia Coli* Constructed by Maximum likelihood Method with nucleotide sequences of 13 reference isolates from GenBank. Current isolates are indicated with black triangle

aggregates in culture supernatants than the wild-type strain. A model for a physical interaction between LPS and *HlyA* is developed, in which LPS with an intact inner core participates(Bauer and Welch, 1997).

Similarly, the rate of *HlyA* secretion in this mutant is unaffected. These findings show that *HlyA* is destroyed more quickly in the mutant's liquid culture media than in the wild-type strains. As a result, LPS may help to protect the released *HlyA* protein from destruction. Early log growth activity produced by cultures was not significantly different from wild-type activity. As growth continued, however, levels of hemolytic activity declined considerably(Stanley *et al.*, 1993).

The HlyA gene in Figure 4 has several mutations in different locations, which may help to explain why some strains do not

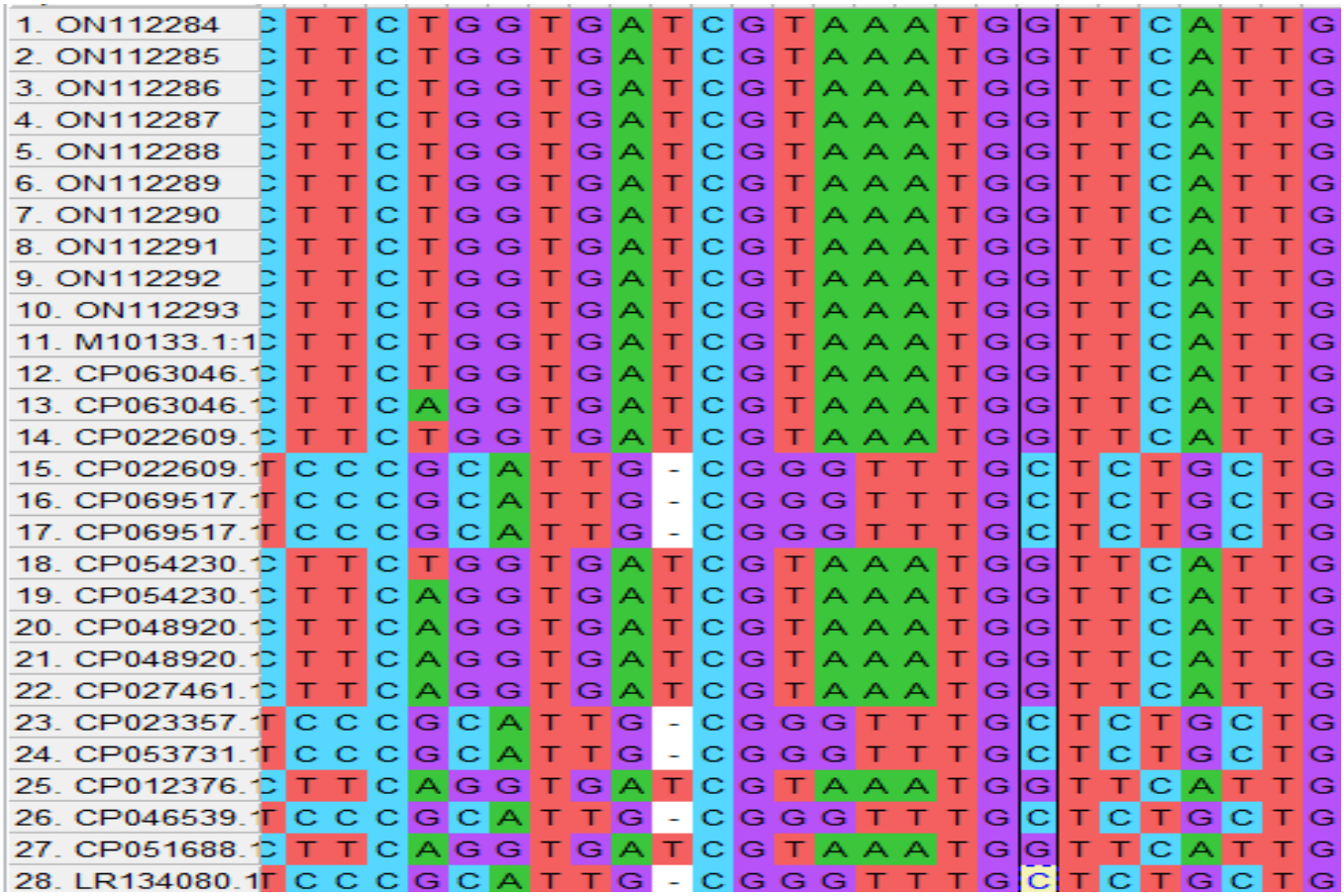

Fig. 4: Alignment of *HlyA* gene of *E.Coli* with the referenced genome from NCBI (gene bank)

Table 2: Sequences producing significant alignments

Descrption	*Scentific name*	*Max Score*	*Total Score*	*Query cover*	*E value*	*Per Ident*	*Acc. len*	*Accession*
E.coli hlyA, Iraq	*Escherchia coli*	1013	3010	100%	0.0	100%	-	ON112292
E.coli (J96) *hlyC*, *hlyA*, *hlyB* and *hlyD* genes coding for chromosal hemolysins C, A,B and D	*Escherchia coli*	4107	4107	100%	o.o	100.00%	8211	M10133.1
Escherchia coli strain KC-Dl-1 chromosome, complete genome	*Escherchia coli*	4053	6807	100%	o.o	99.72%	5305214	CP063046.1
Escherchia coli strain ATCC 700415 chromosome, complete genome	*Escherchia coli*	4049	6803	100%	o.o	99.72%	5237257	CP022609.1
Escherchia coli strain FDAARGOS_1262 chromosome, complete genome	Escherchia coli	4047	6801	100%	o.o	99.67%	5225337	CP069517.1
Escherchia coli strain EcPF14 chromosome, complete genome	*Escherchia coli*	4030	6790	100%	o.o	99.53%	5129852	CP054230.1
Escherchia coli strain SCU-101 chromosome, complete genome	Escherchia coli	3984	6767	100%	o.o	99.11%	5357129	CP048920.1

Descrption	*Scentific name*	*Max Score*	*Total Score*	*Query cover*	*E value*	*Per Ident*	*Acc. len*	*Accession*
Escherchia coli strain 95-3322 chromosal, complete genome	*Escherchia coli*	3978	3978	100%	o.o	99.06%	5095223	CP027461.1
Escherchia coli strain 317 chromosome, complete genome	*Escherchia coli*	3978	3978	100%	o.o	99.06%	5035905	CP023357.1
Escherchia coli strain CP55_Sichuan chromosome, complete genome	*Escherchia coli*	3915	3915	100%	o.o	98.55%	4992761	CP053731.1
Escherchia coli strain GE3, complete genome	*Escherchia coli*	3915	3915	100%	o.o	98.55%	4909965	CP012376.1
Escherchia coli strain FHl_NMBU_10 chromosome, complete genome	*Escherchia coli*	3915	3915	100%	o.o	98.55%	5304684	CP046539.1
Escherchia coli strain SCU-387 chromosome, complete genome	*Escherchia coli*	3909	3909	100%	o.o	98.50%	5329017	CP051688.1
Escherchia coli strain NCTC9082, genome assembly, chromosome 1	*Escherchia coli*	3909	3909	100%	o.o	98.50%	5030213	LR134080.1

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Query 1       GTGGAAGTGCAACTTTTGCTCCGGTTGCTGCAGTAAAACACGAGTATACGTCTGCCGTAC        60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  164    GTGGAAGTGCAACTTTTGCTCCGGTTGCTGCAGTAAAACACGAGTATACGTCTGCCGTAC        223
Query  61     ACATTCATAAGCGATTCTGATGGCTCACTTACGTGGAGAAACTGGAGTGCCGTCTGTTCT        120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224    ACATTCATAAGCGATTCTGATGGCTCACTTACGTGGAGAAACTGGAGTGCCGTCTGTTCT        283

Query  121    TTATTTATTGTTTGTGTTATGTTATAACATATAAAAGAGTATTGTTTGGCATCTGTAACT        180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  284    TTATTTATTGTTTGTGTTATGTTATAACATATAAAAGAGTATTGTTTGGCATCTGTAACT        343

Query  181    TATTGAGAAGGCAAACTGCAGAGTGGTTAATGCAGTAATTGTATTAGCTAAAATTTTGTT        240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  344    TATTGAGAAGGCAAACTGCAGAGTGGTTAATGCAGTAATTGTATTAGCTAAAATTTTGTT        403

Query  241    AATTAAAATTTGTTTGATTGCTAATGGTTTGTGCTGGTTGATGACTGTTAATTCCAGAAG        300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  404    AATTAAAATTTGTTTGATTGCTAATGGTTTGTGCTGGTTGATGACTGTTAATTCCAGAAG        463

Query  301    GCGGTAGTCTGCATTAATATTAGCATTACGGTGACCAGCTTTTATTCCGGCCCCTTCTTT        360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 464      GCGGTAGTCTGCATTAATATTAGCATTACGGTGACCAGCTTTTATTCCGGCCCCTTCTTT        523

Query  361    CATGAAACAACGTATTCCGAAGAATAAGTTTGAGAATGGCAGACGAACTTTTATTATTCT        420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  524    CATGAAACAACGTATTCCGAAGAATAAGTTTGAGAATGGCAGACGAACTTTTATTATTCT        583

Query  421    GCTGAATACAGAAGATATGAATTGTTCTTGTTTATGTCAGATATTCATAACACAGGTATT        480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  584    GCTGAATACAGAAGATATGAATTGTTCTTGTTTATGTCAGATATTCATAACACAGGTATT        643

Query  481    ATGGTTAACTCATAACATTAATTCCTGTATTTTTCTGCTCAATGGCAGCGTCGTATGCAT        540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 644      ATGGTTAACTCATAACATTAATTCCTGTATTTTTCTGCTCAATGGCAGCGTCGTATGCAT        703

Query 541      ATGTTTTTATTTCAAATGAAGCAAGGTGCAGGAAATAAAAATAACCATTTCTTTATGTGA        600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  704    ATGTTTTTATTTCAAATGAAGCAAGGTGCAGGAAATAAAAATAACCATTTCTTTATGTGA        763

Query  601    TCTTTTTATCAATGGAGATAGTATCATTTATAATGAACAGAAACAATCCATTAGAGGTTC        660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764    TCTTTTTATCAATGGAGATAGTATCATTTATAATGAACAGAAACAATCCATTAGAGGTTC        823

Query  661    TTGGGCATGTATCCTGGCTCTGGGCCAGTTCCCCATTACACAGAAACTGGCCAGTCTCTT        720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  824    TTGGGCATGTATCCTGGCTCTGGGCCAGTTCCCCATTACACAGAAACTGGCCAGTCTCTT        883
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Fig. 5: Nucleic acid sequence alignment of 10 *HlyA* gene from bacterial samples with its corresponding reference sequences of the *HlyA* within the *E Coli* genomic DNA sequences

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Query  721    TGTTTGCAATAAATGTATTACCTGCAATACGGGCTAACCAATATGCTTTATTAACCCGGG        780
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  884    TGTTTGCAATAAATGTATTACCTGCAATACGGGCTAACCAATATGCTTTATTAACCCGGG        943

Query  781    ATAATTACCCTGTTGCATATTGTAGTTGGGCTAATTTAAGTTTAGAAAATGAAATTAAAT        840
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  944    ATAATTACCCTGTTGCATATTGTAGTTGGGCTAATTTAAGTTTAGAAAATGAAATTAAAT        1003

Query  841    ATCTTAATGATGTTACTTCATTAGTCGCAGAAGACTGGACTTCTGGTGATCGTAAATGGT        900
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1004   ATCTTAATGATGTTACTTCATTAGTCGCAGAAGACTGGACTTCTGGTGATCGTAAATGGT        1063

Query  901    TCATTGTCTGGATTGCTCCTTTCGGGGATAACGGTGCCCTGTACAAATATATGCGaaaaa        960
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1064   TCATTGTCTGGATTGCTCCTTTCGGGGATAACGGTGCCCTGTACAAATATATGCGAAAAA        1123

Query  961    aaTTCCCTGATGAACTATTCAGAGCCATCAGGGTGGATCCCAAAACTCATGTTGGTAAAG        1020
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1124   AATTCCCTGATGAACTATTCAGAGCCATCAGGGTGGATCCCAAAACTCATGTTGGTAAAG        1183

Query  1021   TATCAGAATTTCACGGAGGTAAAATTGATAAACAGTTAGCGAATAAAATTTTTAAACAAT        1080
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1184   TATCAGAATTTCACGGAGGTAAAATTGATAAACAGTTAGCGAATAAAATTTTTAAACAAT        1243

Query  1081   ATCACCACGAGTTAATAACTGAAGTAAAAAACAAGTCAGATTTCAATTTTTCATTAACAG        1140
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1244   ATCACCACGAGTTAATAACTGAAGTAAAAAACAAGTCAGATTTCAATTTTTCATTAACAG        1303
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Fig. 5 (Contd.): Nucleic acid sequence alignment of 10 *HlyA* gene from bacterial samples with its corresponding reference sequences of the *HlyA* within the *E Coli* genomic DNA sequences.

exhibit its function despite having the gene present in bacteria. Differences in the size and number of hospitals analyzed, the season of sample collection, and medications taken previous to sampling may all be factors in the discrepancy in isolate percentages expressing HlyA. A number of bacterial virulence factors are crucial for causing UTI, and some of these factors seem to be more critical for spreading infection to specific regions of the urinary tract. Examples of virulence factors include the capacity to adhere, the synthesis of urease, and the creation of flagella. (Burall et al., 2004)

CONCLUSION

Sanger sequencing and phylogenic analysis were used to sequence the 16SrRNA and *HlyA* genes. The amount of mutations that occur with the *HlyA* gene, which varies substantially between local and international strains, was highlighted in the study.

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