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Original Research Article

Association between virulence factors and antibiotic resistance in *E.coli* isolated from urinary tract infection patients in Banha University Hospitals, Egypt

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Abstract

This study aimed to assess the antibiotic resistance pattern among *E. coli* isolated from patients with UTI, to detect the presence of virulence factors and biofilm formation and to evaluate the relation between virulence factors and antibiotic resistance. A cross sectional study was performed in Banha University Hospitals, Egypt from May to November, 2020. Urine samples were obtained from included patients. Vitek-2 was used for identification of isolates and perforrming antimicrobial suscebtipility testing. Congo red agar method was used to detect biofilm formation. Isolated *Escherichia coli* strains were screened for harboring virulence genes; *Pap C, Fim H, Sfa, Afa, Hly A,* and *Cnf1* using multiplex PCR. *E.coli* isolates showed highest resistance against ampicillin 86(96.6%) followed cefazolin 85(95.5%), while the most susceptibility was to both imipenem and meropenem 85(95.6%). Sixty two (67.7%) isolates were multidrug-resistant. Biofilm formation was detected in 54(60.7%) isolates. *FimH* has the highest prevalence 63(70.8%) among *E. coli* isolates, followed by *Pap C* 37(41.6%) and the least was *Cnf 1* genes 2(2.2%). The prevelance of the *FimH* gene was markedly higher in the strains resistant to ampicillin, cefazolin, ceftazidime and ceftriaxone in comparison to the susceptible strains. The *Pap C* gene was related to resistance to ceftazidime, amoxicillin/clavulanic acid and Piperacillin/ Tazobactam. The *Cnf1* gene was related to resistance to amoxicillin/ clavulanic acid and nitrofurantoin. There was a statistically significant difference between biofilm production and resistance to ampicillin, ceftriaxone, ceftazidime and ciprofloxacin. In conclusion multi drug resistant *E. coli* showed high prevelance among isolates 62(67.7%) which is a significant clinical challenge. The highest prevalence of virulence genes among the *E coli* strains such as *FimH* and *Pap*C, suggesting their importance in pathogenesis of UTI caused by *E coli*. Relation between the virulence factors and antimicrobial resistance should be periodically evaluated in each health care facilities.

Keywords: Biofilm, *Escherichia coli,* UTI, Virulence factors

INTRODUCTION

Escherichia coli (E coli) is one of the most common etiological agents for urinary tract infections (UTI) (Farell D. et al., 2003). There are two key groups of *E. coli* virulence factors that affect its pathogenicity; which include virulence factors that are synthesized inside the cell and delivered at the action site and virulence factors

expressed on the cell surface (Matute AJ, 2004). Surface virulence factors (adhesins) are the most common factors in *E.coli* causing UTI infections, including; P fimbriae,S fimbrial, A fimbrial and type 1 fimbriae which are coded by *Pap* genes, *Sfa* genes, Afa genes and *FimH* respectively (Servin AL., 2005) the other important types of virulence factor in *E. coli* is toxins which include a-hemolysin (*HlyA*) and cytotoxic necrotizing factor which are encoded by the Hly gene and CNF15 genes (Slavchev G et al, 2009 and Firoozeh F. et al., 2014). Biofilm-producing bacteria create a matrix made up of proteins, polysaccharides and extracellular DNA, which have many advantages for bacterial species including persistence of bacteria and promoting their growth, Moreover the bacteria become more resistant to the antimicrobial substances (Neupane S et al., 2016)

E. *coli* causing UTI was historically treated with oral antibiotics such as trimethoprim-sulfamethoxazole, cephalosporins and fluoroquinolones. Recently, bacteria developed antibiotic resistance because of wide spread and improper use of such antibiotics (Chen Y. et al., 2013). Therefore, physicians should be aware of appropriate antibiotics for effective management of UTI patients in each geographical region (Yadav K.et al., 2015)

Antimicrobial resistance has emerged in different antimicrobial groups, including penicillins, cephalosporines, carbapenems, sulfonamides, aminoglycoside, macrolides, and polymyxins, due to misuse and overuse in human therapeutic applications, as well as as growth promoters in livestock (Emody L et al., 2003). The failure to monitor the spread of multidrug (MDR), extensive drug resistance (XDR) and pan resistance would have increased rates of morbidity and mortality worldwide (Zhanel G et al., 2006)

The aim of the study was to assess the antibiotic resistance pattern among *E. coli* isolated from patients with UTI, to detect the presence of virulence factors and biofilm formation and to evaluate the relationship between virulence factors and antibiotic resistance.

MATERIALS AND METHODS

Study design

A cross sectional study was performed in urology and microbiology departments of banha university hospitals, Egypt from May to November, 2020.

Patients with urinary tract infections were included in the study and a consent for participation was collected from each patient.

Specimen collection

Urine samples were obtained from all UTI patients into sterile disposable container or sterile urine bags for adults and children respectively, then were sent to laboratory immediatly.

Bacterial isolation and identification

Each collected urine specimens were streaked on CLED, Maconkey and blood agar(oxoid, UK) and incubated aerobically for 24 hrs at 37°C. UTI is charachtarized by the presence of single organism with bacterial counts to ≥ 10^5 CFU/ml.

E Coli strains were detected by morphology of colony, Gram stained films and confirmed using the Vitek-2 identification machine (BioMerieux, France) by the gram negative (GN) identification cards as per the manufacturer's guidlines. Pure isolated *E. coli* strains were stored at − 80 °C in 20% glycerol until used.

Susceptibility testing detection

Antimicrobial susceptibility testing (AST) was done by the Vitek-2 machine (BioMerieux, France), according to manufacturer's instructions. The antimicrobials used were included in the AST cards that contained penicillins group: ampicillin, amoxicillin-clavulanate and piperacillin/ tazobactam; cephalosporins group: ceftazidime, cefazolin, ceftriaxone and cefepime; carbapenems group: meropenem and imipenem, inhibitors of folate pathway: nitrofurantoin trimethoprim-Sulfamethoxazole; aminoglycoside: amikacin and gentamicin,and fluoroquinolone: ciprofloxacin (Ramírez-Castillo, F. et al., 2018). When isolates were resistant to minimum three or more antibiotic classes, they were classified as MDR. (Magiorakos P et al., 2012).

Assessement of biofilm formation

Biofilm formation was detected using the congo red agar method (CRA) (Gilbert E., 2004). CRA medium was made using 37 g/L brain heart infusion broth, 50 g/L sucrose, 10 g/L agar, and 8 g/L Congo red indicator (Oxoid, UK). The Congo red stain was made as a concentrated aqueous solution and sterilized apart from the other medium components (121 °C for 15 minutes).Later, at 55 °C, it was applied to the sterilized brain heart infusion agar with sucrose. Congo red agar plates were then subcultured with isolates and aerobically incubated for 24 hours at 37 °C., Biofilm formation was reported with black, dry colonies, while non-biofilm producing strains was detected as red or pink shiny colonies. *E. coli* ATCC 25922 served as positive control while *Staphylococcus aureus* served ATCC 25932 as negative control.

Table 1. Antibiotic susceptibilty pattern among UTI strains

Antibiotic	Susceptible	Resistant
Ampicillin	3(3.4%)	86(96.6%)
Amoxicillin-Clavulanate	69(66.3%)	30(33.7%)
Piperacillin/Tazobactam	83(93..3%)	6(6.7%)
Cefazolin	4(4.5%)	85(95.5%)
Ceftazidime	29(32.6%)	60(67.4%)
Ceftriaxone	47(52.9%)	42(47.1%)
Cefepime	60(67.4%)	29(32.6%)
Meropenem	85(95.6%)	4(4.4%)
Imipenem	85(95.6%)	4(4.4%)
Nitrofurantoin	60(66.2%)	39(43.8%)
Trimethoprim-Sulfamethoxazole	24(27%)	65(73%)
Amikacin	70(87.7%)	18(14.6%)
Gentamicin	44(49.4%)	45(50.6%)
Ciprofloxacin	69(77.5%)	20(22.5%)

Multiplex Polymerase Chain detection of Virulence Genes

Isolated *Escherichia coli*strains were screened for harboring virulence genes which include: *FimH, Pap C , Sfa, Afa, HlyA, and Cnf*1 using multiplex PCR technique (Abd El-Baky R. et al., 2020)

Genomic DNA extraction was done as per the manufacturer's guidelines of the QIAamp DNA Mini Kit (Qiagen, GmbH, Germany). Apure culture of each strain was mixed with 70µl DNase-free water and heated for 10 minutes at 95°C. All of the genes involved were amplified using Dream Taq PCR Master Mix (Fermentas, US) and the primers used were commercially constructed as follows: *Fim-H-F* (TGTACTGCTGATGGGCTGGTC), *SfaF (*CTCCGGAGAACTGGGTGCATCTT AC*), Afa-F (*GCTGGGCAGCAAACTGATAACTCTC*), HlyAF (*AACA-AGGATAAG CACTGTTCTGGCT*), PapCF* (GACGGCA-CTGCTGCAGGGTGTGGCG), *Cnf1-F* (AAGATGGAG-TTTCCTATGCAGGAG). The reaction mixture volume was 25 µl prepared using {12.5 µl Dream Taq Green PCR Master Mix ,1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 1 µl of bacterial lysate, and 9.5 µl of nuclease-free water}. A positive and negative PCR control was done. Initial denaturation for 5 minutes at 95°C was then 40 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at temperatures defined for each primer, then extension for 1 minute at 72°C. Next a 5-minute final extension stage at 72°C was done. TECHNE® Ltd. peltier thermal cycler (Germany) was used for amplification.The separation of PCR products were done by using agar gel electrophoresis stained with ethidium bromide 0.5 µg/ml

Data analysis

Data obtained were analysed using SPSS version 17 (Chicago software). Data was interpreted as numbersand percentages. _ Z ˝ test for 2 variables and ˝ χ2 _(Chi square) test for more than two were used as testsof significance. P value of <0.05 was considered statistically significant.

RESULTS

Sixty four (71.9 %) of the 89 UTI patients were females, while 25 (28.1 %) were males, aged from 16 to 82 years with median age 39. and the highest incidence was in age group (20-40).

E. Coli isolates resistance pattern was showed in table 1, the highest resistance was against ampicillin 86 (96.6%) followed cefazolin 85(95.5%), cotrimoxazole 65(73%) and ceftazidime 60(67.4%) while the highest susceptiblity was to both imipenem and meropenem 85(95.6%) followed by piperacillin/tazobactam 83 (93.3%), amikacin 70(87.7%) and ciprofloxacin 69(77.5%). Seventy seven (86.5%) strains were resistant to only one antimicrobial agent group, 72(80.9%) strains were resistant to two antimicrobial agent groups or more and 62(67.7%) of strains were MDR.

Table 2 and Figure 1 showed that out of 89 *E. coli* clinical isolates, 54(60.7%) had the ability to synthesize biofilm, while out of the 62 MDR E. coli strains, 45(72.3%)) were biofilm producers.The percentage of biofilm production was increased in MDR *E. coli* than other involved *E. coli* strains. Figure 1

PCR was performed to detect virulence genes, as showed in table 3 *fimH* had the highest prevalence 63(70.8%), followed by *Pap*37(41.6%), both *Sfa and HlyA was* 9 (10.1%) and the least was *Cnf* genes2 (2.2%). The most frequent coexesting genes detected in some isolates were *PapC* plus *fimH* 35(39.3%). while 4(4.5%) had no virulence factors. Virulence genes were highly detected in MDR strains out of the 62 strains, *FimH* was

Table 2. Biofilm formation among totally isolated bacteria and MDR isolates

Biofilm formation	Total isolates = 89	MDR isolates = 62
Positive	54(60.7%)	45(72.3%)
Negative	35(39.3%)	17(27.4%)

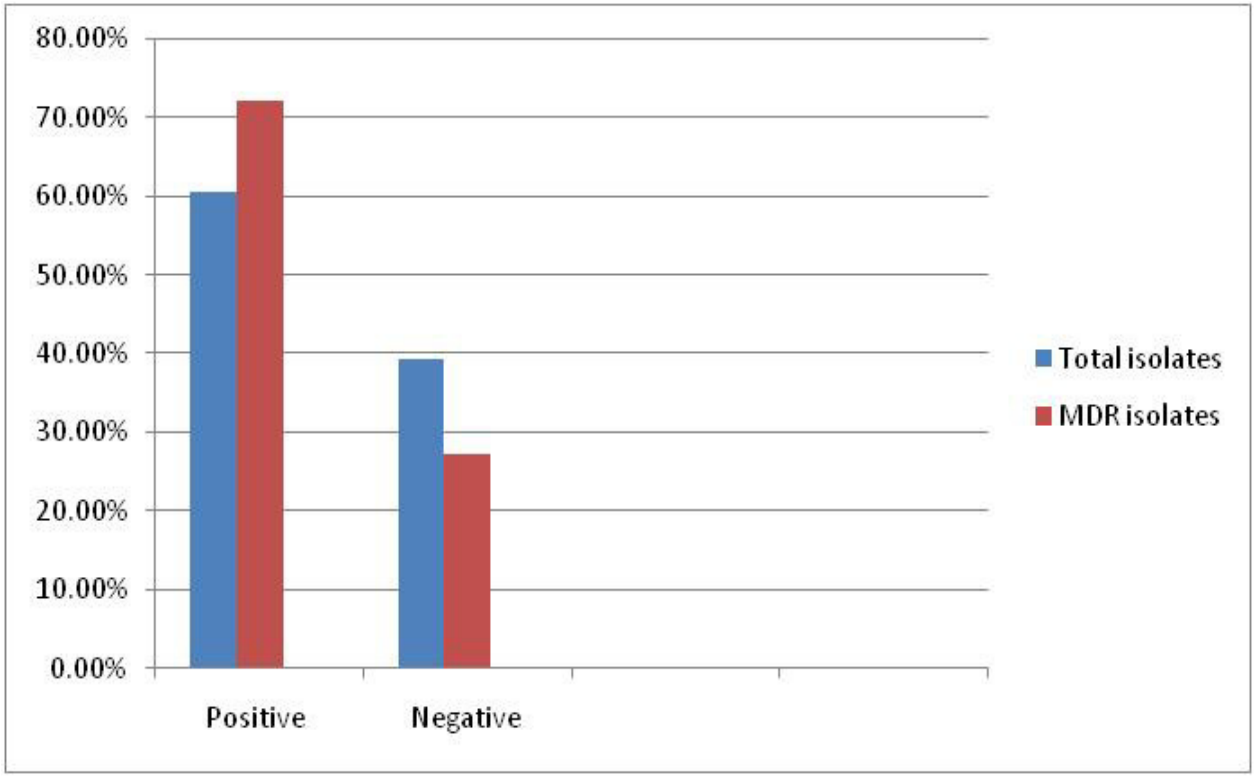

Figure 1. Biofilm formation among totally isolated bacteria and MDR isolates

Table 3. Prevelance of virulence factors among totally isolated bacteria and MDR isolates

Virulence genes	Total isolates *n* = 89	MDR *n* = 62
FimH	63(70.8%)	43 (69.4%)
SfaS	9 (10.1%)	6 (9.8%)
Afa	8 (8.9%)	5 (8.1%)
PapC	37 (41.6%)	27 (43.5%)
HlyA	9 (10.1%)	7 (11.3%)
Cnf1	2 (2.2%)	1 (1.6%)
Novirulence factors	4(4.5%)	-

43 (69.4%)followed by *PapC* 27 (43.5%), *HlyA*7 (11.3%) and the least was *Cnf1* genes 1 (1.6%).

Table 4 Showed that the prevelance of the *FimH* gene was markedly higher in the strains resistant to ampicillin, cefazolin, ceftazidime and ceftriaxone in comparison to the susceptible strains. The *PapC* gene was related to resistance to ceftazidime, amoxicillin/clavulanic acid and Piperacillin/Tazobactam. The *Cnf1gene* was related to resistance to amoxicillin/clavulanic acid and nitro-furantoin. There was association between The *HlyA* gene and amoxicillin/clavulanic acid resistance. There were no association detected between any of the virulence factors and cefepime, meropenem, imipenem, trimethoprim-Sulfamethoxazole, amikacin, gentamicin and ciprofloxacin There was a statistically significant difference between biofilm production and resistance to ampicillin, ceftriaxone, ceftazidime and ciprofloxacin.

Table 4. Association between virulence factors and antibiotic resistance:

Antibiotics	Virulence genes						Biofilm
	FimH	*PapC*	*Afa*	*SfaS*	*Cnf1*	*HlyA*	
Ampicillin	0.053	0.261	*0.079	0.331	0.205	0.564	*0.021
Amoxicillin-Clavulanate	0.881	*0.059	0.005	0.107	*0.006	*0.043	0.764
Piperacillin/Tazobactam	0.87	*0.009	0.234	0.154	0.237	0.340	0.516
Cefazolin	*0.057	0.788	0.151	0.879	0.341	0.465	0.543
Ceftazidime	*0.054	*0.041	*0.022	0.346	0.655	0.423	*0.008
ceftriaxone	*0.076	0.356	0.287	0.451	0.342	0.092	*0.098
Cefepime	0.312	0.873	0.434	0.438	0.657	0.451	0.685
Meropenem	0.231	0650.	0.478	0.309	0.238	0.351	0.306
imipenem	0.207	0.331	0.230	0.128	0.284	0.235	0.318
Nitrofurantoin	0.107	0.165	0.785	0.332	*0.034	0.656	0.760
Trimethoprim-Sulfamethoxazole	0.324	0.543	0.540	0.981	0.765	0.342	0.548
Amikacin	0.213	0.415	0.512	0.122	0.143	0.367	0.165
Gentamicin	0.524	0.231	0.123	0.577	0.651	0.227	0.129
Ciprofloxacin	0.334	0.212	0.541	0.650	0.144	0.277	*0.029

*P values <0.05 was statistically significant.

DISCUSSION

The existence of the bacterial virulence factors has been shown to infleuence the intensity and frequency of any infection caused by pathogenic microorganisms. Furthermore, the patient's medical conditions and other host factors also influence the presence of infection (Jauréguy F. et al., 2007; Dale AP, Woodford N. 2015)

In this study sixty four (71.9 %) of the 89 UTI patients were females, while 25 (28.1 %) were males, aged from 16 to 82 years with median age 39 and the highest incidence was in age group (20-40). Dadi et al, 2020 reported similar results that 34% of studied cases were males and 66% were females due to short wide female urethra. He also reported that there was increased incidence of urinary tract infections in the age groups ranged from 26 to 45 years as they include the most sexually active age groups.

In the current study ampicillin 86 (96.6%) showed the highest resistance followed cefazolin 85(95.5%), cotrimoxazole 65(73%) and ceftazidime 60(67.4%) while the highest susceptiblity was to both imipenem and meropenem 85 (95.6%) followed by piperacillin/ tazobactam 83 (93.3%), amikacin 70(87.7%) and ciprofloxacin 69 (77.5%). In an Egyptian study by Elsayed et al, 2017 ,he reported similar pattern of antimicrobial resistance in *E coli* isolated from UTI patients, ampicillin resistance was 95%, sulphamethoxazole/trimethoprime resistance was 69%, cephalothin resistance was 93% and Imipenem resistance was 2%. El-Kholyet al.2020 also, reported that *E. coli* showed high susceptiblity to amikacin (94.1%), imipenem (90.4%) and meropenem (83.6%). In this work 62 (67.7%) of isolates were multidrug-resistant. This was similar to Arslan et al., 2005 who reported in a different study that 60% of isolates from UTI were MDR.On the other hand Elsayed et al, 2020 reported a higher incidence of multidrug resistance as 95% of the isolates.

In the current study out of 89 *E. coli* clinical isolates, 54(60.7%) had the ability to synthesize biofilm, while out of the 62 MDR *E. coli* strains, 45(72.3%)) were biofilm producers.The percentage of biofilm production was increased in MDR *E. coli* than other involved *E. coli* strains. Sevanan et al., 2011 also by using same method, showed that 59.4% strains produce biofilm. Increased biofilm formation was similary noted in 67.5% isolates of *E. coli* in a research performed by Sharma et al,2009 using TCP method. Different studies have also shown that the biofilm prevalence among uropathogenic *E. coli* was between 60% to 70%. (Saroj G et al., 2012 and Karigoudar R. et al., 2019). In agreement with Sharma et al,2009 there was a significant association between biofilm synthesis and resistant to different antibiotics.

The key step in the development of UTI is the adhesion by *FimH, Pap C, Sfa, Afa* pilli to urinary epithelial cells, leading to *E coli* invasion and colonization of urinary tract. *HlyA* secreted by *E.coli* isinvolved in damage of tissues and local immune responses dysfunction (Momtaz H. et al., 2013). In this study PCR was performed to detect virulence genes, *FimH* was observed to have the highest prevalence, 63(70.8%), followed by *Pap C,* 37 (41.6%), both *Sfa and Hly* was 9 (10.1%) and the least was *Cnf 1*genes 2(2.2%). Dadi et al, 2020 also reported that *FimH* had the highest prevelance (82%) among *E. coli* virulence genes,. *FimH* also detected in 72% of *E coli* isolates in Egypt as reported by Hassan et al.,2011. Also other published studies (Gao Q et al., 2017, Tabasi M et al., 2015 and Jadhav S. et al, 2011) reported highest presence of *FimH* gene in the isolated strains. Jalali et al. (2015) reported that 46% of isolates had *Pap C* gene, and Tarchounaet al. (2013), who found that 41% of isolates had *PapC* positive gene. Jalali et al. (2015) found high

percentage of *HlyA* positive gene(47%). Prevalence of these genes differs according togeographical distribution, phylogenetics, and clinical presentation (Oliveira F. et al.,2011). Discripancies in the virulence genes prevelance was recorded all over the world (Abe C et al., 2008). In this work there was 4(4.5%) strains had no virulence factor. This is in agreement with the findings of Tarchouna et al., 2013, who isolated six (6%) strains of *E. coli* that were suscebtible to all antibiotics while it was negative for virulence genes

The prevelance of the *FimH* gene was markedly higher in the strains resistant to ampicillin, cefazolin, ceftazidime and ceftriaxone in comparison to the susceptible strains. The *PapC* gene was related to resistance to ceftazidime, amoxicillin/clavulanic acid and Piperacillin/Tazobactam. The *Cnf1gene* was related to resistance to amoxicillin/clavulanic acid and nitrofurantoin. there was association between The *HlyA* gene and amoxicillin/clavulanic acid resistance. Derakhshandeh et al., 2015 also noted that on studying antibiotic resistance in the virulent isolates, there was association noticed as the strains harboring the virulence genes had higher resistance to several antibiotics such as ampicillin, cephalosporins and nitrofurantoin.

There was no association detected between any of the virulence factors and cefepime, meropenem, imipenem, trimethoprim-Sulfamethoxazole, amikacin, gentamicin and ciprofloxacin resistance.Moreno et al. 2006 and Rijavec et al. 2008 reported low virulence capability in resistance to quinolones and trimethoprim-sulfameth-oxazole compared to susceptible strains,Virginioet al., 2020 noted that the development of antimicrobial resistance can be correlated with a decrease in a microorganism's virulence levels as reported also in various studies. A possible explanation for these results could be that when the strain is resistant to quinolones or other antibiotics by a mutation, this mutation may affect the virulance gene. (Johnson, J.et al., 2005)

Analyzing of biofilm production with antimicrobial resistance showed a significant statistical difference with resistance to ampicillin, ceftriaxone, ceftazidime and ciprofloxacin. This similar to the study of Soto etal.,2007 who reported a relationship between biofilm formation and resistance to quinolones and cephalosporins. Biofilm-forming isolates were also found to have a higher level of antibiotic resistance than non-biofilm producing isolates as reported in previous studies. (Tadepalli S et al., 2016 and Makled A et al., 2017). Nitrofurantoin and amikacin resistance rate is low in association with biofilm production and could be effective against biofilm producers (Ghosh P. et al., 2016 and Bagel, S et al., 1999)

CONCLUSION

Multi drug resistant *E.coli* showed high prevelance among isolates 62 (67.7%) which is a significant clinical challenge urging continuous monitoring of antibiotic resistance and decreasing the improper adminstration of antibiotics. The highest prevalence of virulence genes among the *E coli* strains such as *FimH* and *Pap C*, suggesting their importance in pathogenesis of UTI caused by *E coli* .The relation between these virulence factors and antimicrobial resistance should be periodically evaluated in each health care facilities.

Authors Contribution

The two authors worked together to conduct this work. Author ESHK planned and designed the research, prepared the protocol, obtained the samples, engaged in the interpretation and review of the findings, drafted and critically revised the manuscript. Author HWS was involved in the study's preparation and design, as well as sample collection and interpretation of the results. the final manuscript read and approved by both authors .

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