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Genetic Diversity of Some *Proteus mirabilis* Strains Isolated from Gastric Discorded Egyptian Patients.

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Abstract: *Proteus mirabilis* (*P. mirabilis*) is a member of the *Enterobacteriaceae* family that is linked to the increased risk of pathogenicity and acquisition of antibiotic resistivity. Moreover, *P. mirabilis* is common in urinary tract infection, it is also considered a potential gut pathogen that has an etiological role in Inflammatory bowel disease (IBD). The IBD that represented any inflammation occurred prolonged the gastrointestinal tract. This study includes a clinical survey for a group of sixty-eight patients with different gastric disorder diseases, aged ranges between 5 and 76 years, (61.77%) were females and (38.23%) were males. Only twenty-eight (41.18%) of patients were positive for *P. mirabilis* infection. Our results demonstrated a significant relation between *P. mirabilis* infection and colitis ($P > 0.05$). The Molecular analysis indicated that from the 28 isolates only four strains of *P. mirabilis* were chosen due to their distinction; they were isolates 2, 10, 16 and 23. More importantly, *P. mirabilis* isolate 10 was the most frequent one. Also, the in-vitro antibiotic sensitivity of the four isolates indicated their resistance to β-lactams, fluoroquinolones, sulfonamides and chloramphenicol classes of antibiotics. In conclusion, *P. mirabilis* is linked to gastric disordered diseases prevalent among Egyptian patients.

keywords: *Proteus mirabilis*, Gastrointestinal Discorders.

1.Introduction

Despite the fact that *P. mirabilis* familiarity in clinical settings as an uropathogen, the complete genome sequencing revealed different virulence factors that boost its

colonization also in the gut environment to stimulate gastrointestinal diseases **[1,2]**. The Gut mucosa is a convenient environment induce expression of of flagillin (FLA) gene appropriately subunits decomposition. Swarming behaviour initiated by intestinal amino acids metabolites such as choline, glutamine and polyamines (putrescine) either under aerobic or anaerobic conditions **[3-6]**. A 17 highly conserved fimbrial operons, and cellular invasion pathways in this bacterium help in the gastrointestinal epithelial adhesion **[7,8].** Moreover, virulence factors promote extensive colonization in host cells immune system **[8-10]**. In addition, the bacterium's enzymatic activity also is known to play a dual role in pathogenicity and etiology of inflammatory bowel diseases (IBD). Moreover. The overexpression of urease in concert with other gut microbes trigger ammonia production and nitrogen flux that cause a shift in the metabolic pathways and leads to synthesis of alternative byproducts which promote mutation within gut microbiota community **[11-15]**. The proteolytic activity boost immune evasion, ZapA metalloprotease represent defense factor against innate immune response degrade immunoglobulin (Igs) and other associated peptides as defensins **[16-18]**. Hemolysin activity of *P. mirabilis* lyse innate immune cells, induces interleukin IL-1β release and increase inflammation **[16-20]**.

Early literatures highlited the correlation between Prevalence of *P. mirabilis* and

gastrointestinal disorders as diarrheal diseases **[21,22]**. Crohn's disease and ulcerative colitis **[23,24]**. Recent advanced studies stated alternation metagenomic profile within IBD and stated dominance of *P. mirabilis*, particularly during the developed stages **[25-27]**. Pioneer Garrett et al' in-vivo trials since 2007, illustrated ulcerative colitis is attributed to immune dysfunction is induced by the synergistic interaction between both *P. mirabilis* and *Klebsiella pneumonia* in TRUC mice that may be developed into colorectal cancer **[28-30]**. Also, other risk factors could trigger IBD as age, gender and smoking either direct or passive smoking, such microbial and external factors alter immune response and initiate epigenetic mutations **[31-34]**. In this study we isolated *P. mirabilis* strains from gastrointestinal referral and follow-up clinics. Moreover, we addressed the correlation between *P. mirabilis* infection in gastrointestinal disorders and the demographic analysis for patients with regard to age, gender and smoking habit among the participants in the study.

2. Materials and methods

Study Subject and Sample Collection: All clinical feces (68 samples) were collected from patients diagnosed to have distinctive gastric disorders with severe diarrhea, gastritis, colitis, gastric, duodenal ulcers and cancer attended Dakahlia Governorate hospitals. Each sample was suspended in 3 ml of Selenite Cystine Broth Base media (Selenite Cystine Broth: Oxoid CM0699) and Sodium Biselenite (Sodium Biselenite: Oxoid CM0395 .(

Bacterial Isolation and Primary Identification: The samples were transported within 2 h and the diluted specimens were spread over Brain Heart Infusion plates (Brain Heart Infusion agar base: Oxoid CM1136) supplemented with 5 to 7% defibrinated human blood) and incubated at 37º C for 48 h.Whitish gray colonies with swarming phenotyping suspected of being Gram-negative rods were purified and subjected for further examination [35]. The primary identified P. mirabilis isolates were stored in Tryptone Soya Broth (TSB: Oxoid: CM0129) with 20% (W/V) glycerol at -80o C until needed for further studies.

a- Biochemical Tests: These were performed according to Bergey's Manual (2005) [35] ; it included urease test, oxidase, test and catalase test.

b- Molecular Identification: The molecular methods used in identification of the local clinical isolates included 1) examination of the total cellular protein profile, 2) 16S rRNA characterization and sequencing as detailed below.

1. Protein banding patterns:Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel was used to fractionate the cellular proteins and examine their patterns as originally recommended by (Laemmli, 1970) [36] . The cell pellets from overnight cultures were treated with double strength breaksge buffer, boiled for 90seconds and loaded onto the denatured gel for the electrophoresis. Upon complation the gels were removed and stained with Coomassie Brilliant Blue R-250 (Sigma) at room temperature for overnight with gentle shaking and destained in 40 % (w/v) methanol: 10% (w/v) acetic acid .
2. 16s RNA Genotyping and sequencing The whole genomic DNAs from selected four distinctive strains were purified using DNA isolation Kit (Thermo Fisher, USA) following the manufacturer's instructions.. Two oligonucleotides primers were used to genotype the four local isolates of P. mirabilis, the sequence of the forward primer: 5′-GAGTTTGATCCTGGCTTAG-3′ and reverse primer R: 5′-GGTTACCTTGTTACGACTT-3′. Specific DNAs were amplified as follow: initial denaturation step for 30 s at 94ºC followed by thirty-seven cycles for primer annealing for 60 s at 51ºC and strands extension for 5 minutes at 72º C [37]. The PCR products were purified by following the manufacturer's instructions of PCR clean-up kit (QIAGEN: QIAquick PCR Purification Kit). The cleaned PCR Products were sequencedusing the BigDye® Terminator v3.1 Cycle Sequencing Kit and the 3500 Genetic Analyzer Applied Bio-systems
3. Bioinformatics and phylogenetics: Sequencing data were used to identify strains by matching them with those

deposited in the GenBank they submitted to the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information website (NCBI) http://www4.ncbi.nlm.nih.gov. Phylogenetic analysis was done using Phylogeny.fr.

4. Antimicrobial susceptibility: Antimicrobial susceptibility test was performed by the disc diffusion method where each strain was grown on brain heart infusion blood agar media then antibiotic discs were placed onto the bacterial culture [39]. The selected antibiotics (Oxoid) were: Amoxicillin-clavulanate (30 µg) , Azithromycin (15 µg), Norfloxacin (10 µg) , Imipenem (10 µg) , Chloramphenicol (30 µg), Ceftolozane Tazobactam (40 µg), Meropenem (10 µg), Cefoperazone (30 µg), Ampicillin-Sulbactam (20 µg) and Piperacillin-Tazobactam (40 µg). The diameter of inhibition zones was measured after incubation at 37o C for 24 h to CLSI standards (2012) [40 .[

1. Statistical Analysis The hypothesis being tested gastrointestinal diseases and associated bacterial pathogen(s). Two patient groups among P .mirabilis positive infection and negative cases at confidence level 95%. Thus, statistical significance was accepted for p <0.05. The analyses were performed via Graph-Pad Prism System, version 5.01 (Graph-Pad Software, La Jolla, USA) and commercial Microsoft Excel version 10 .

3. Results and Discussion

2. Isolation and Identification of Proteus mirabilis (P. mirabilis): Bacterial Characterization: The 68 isolates were subjected to phenotyping characterization, 28 isolates were identified as Gram-negative bacilli, positive urease, catalase and negative oxidase , these bacteria were identified as P. mirabilis. Twenty-eight patients (41.18%) were found to be infected with P. mirabilis among sixty-eight gastrointestinal counselling patients. Exactly, 26 patients (38.23%) were males and 42 (61.77%) were females aged between 5 and 76 years. Their medical history sheet showed that, 23 patients (33.82%) were smokers and

showed several symptoms including; severe diarrheal 14 patients (20.59%), gastritis 22 patients (32.35%), duodenal ulcer 9 patients (13.24%), colitis 15 patients (22.05%), gastric cancer 8 patients (11.76%). Only 40.48% of female were positive P. mirabilis infection, 10 (90.91%) of P. mirabilis male patients were smokers. P. mirabilis has significant correlation with colitis incidence 11 (73.33%) at (P > 0.05). No significant differences (P > 0.05) among P. mirabilis positive patients with regard to gender, age and smoking life habit (Table 1 .(

Protein Profile: Proteins of 28 primary idetification positive P. mirabilis were purified and resolved on three SDS-PAGE protein gels. All protein yield produced districted patterns with molecular weight ranged between 20 and 212 KDa. A single strain was selected as a representative of each protein profile group. Different strains from each group were resolved on gel no. (4), it shows that there were four diverse strains. Group 1 includes strains no. (2-9-17-36-52-7), group 2 includes strains no. (23 - 56), group 3 includes strain no. 10 and group 4 includes strain no 16. Protein profile revealed that there were four distinctive groups and a single strain was selected to represent each group as following; P. mirabilis 2, P. mirabilis 10, P. mirabilis 16 and P. mirabilis 23. Only P. mirabilis 10 was the commonest isolate .

Table (1) The prevalence of P. mirabilis among the 68 patients according to gender, life style, diagnosis and age.

Characteristics Patients Positive P. mirabilis Chi-square with Yates' correction

X2 P -Value Odds Ratio

)Risk Factor\(

N: total number of patients; N+: total number of patients with P. mirabilis infection; *: Statistical significance, NS: non- Statisticant

Table (1) The prevalence of *P. mirabilis* among the 68 patients according to gender, life style, diagnosis and age.

N: total number of patients; N^+: total number Statistical significance, NS: non- Statisticant of patients with *P. mirabilis* infection

Characteristics	**Patients** N= 68(%)	**Positive *P. mirabilis*** N^+ =28(%)	**Chi-square with Yates' correction** X^2	***P* -Value**	**Odds Ratio (Risk Factor)**
Gender:					
MaleFemale	26 (38.24%) 42 (61.76%)	11 (42.31%) 17 (40.48%)	0.011	0.917 (NS)	1.078
Life Habit: Smoking	23 (33.82%)	10 (43.48%)	0.0002	0.988 (NS)	1.154
Diagnosis:					
Gastritis	22 (32.35%)	10 (45.45%)	0.054	0.816 (NS)	1.296
Severe Diarrheal	14 (20.59%)	2 (14.28%)	3.958	0.047 (NS)	0.180
Colitis	15 (22.06%)	11 (73.33%)	6.601	0.0102 (*)	5.824
Ulcer	9 (13.24%)	4 (44.44%)	0.3334	0.564 (Ns)	1.957
Cancer	8 (11.76%)	1 (12.5%)	1.883	0.17 (Ns)	0.175
Age in years:					
5:15	20 (29.41%)	9 (45%)	**Chi-square X^2=2.655, 6** **P value=0.8508** **NS**		
16:25	2 (2.95%)	1 (50%)			
26:35	10 (14.71%)	6 (60%)			
36:45	6 (8.82%)	2 (33.33%)			
46:55	6 (8.82%)	2 (33.33%)			
56:65	17 (25%)	6 (35.29%)			
66:75	7 (10.29%)	2 (28.57%)			

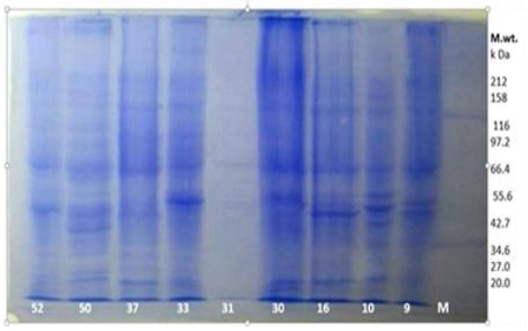

Figure (1) Gel; Lane1: isolate 52 , lane 2 : isolate 50, lane 3 : isolate 37 , lane 4 : isolate 33 , lane 5 : isolate 31 , lane 6: isolate 30 , lane 7 : isolate 16 , lane 8 : isolate 10 , lane 9: isolate 9 , lane10 : protein ladder

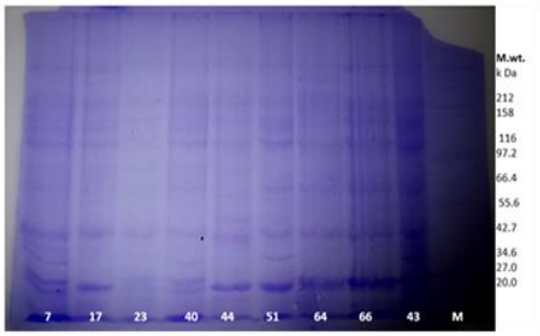

Figure (3) Gel 3; Lane 1: isolate 7 , lane 2 : isolate 17, lane 3 : isolate 23 , lane 4 : isolate 40 , lane 5 : isolate 44 , lane 6: isolate 51 , lane 7 : isolate 64 , lane 8 : isolate 66 , lane 9: isolate 43 , lane 10 : protein ladder

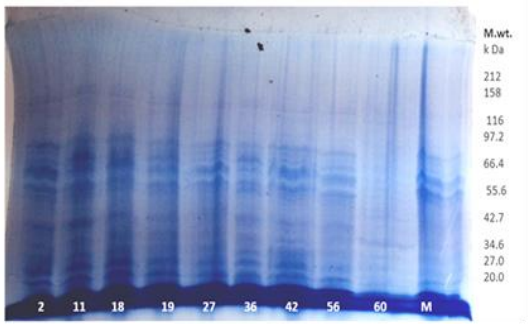

Figure (2) Gel ; Lane 1: isolate 2 , lane 2 : isolate11, lane 3 : isolate 18 , lane 4 : isolate 19 , lane 5 : isolate 27 , lane 6: isolate 36 , lane 7 : isolate 42 , lane 8 : isolate 56 , lane 9: isolate 60 , lane 10 : protein ladder

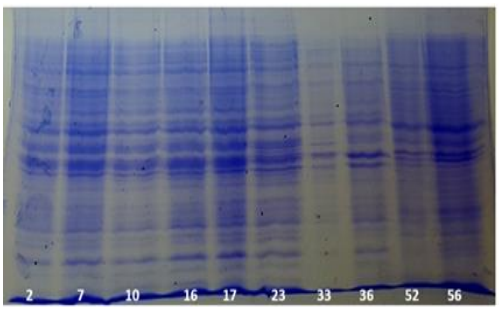

Figure (**4**) Gel 4; Lane 1: isolate 2 , lane 2 : isolate 7, lane 3 : isolate 10 , lane 4 : isolate 16 , lane 5 : isolate 17 , lane 6: isolate 23 , lane 7 : isolate 33 , lane 8 : isolate 36 , lane 9: isolate 52 , lane10 : isolates 56 .

Antimicrobial susceptibility: All of the four *P. mirabilis* isolates (2, 10, 16 and 23) were tested for their susceptibilities to fifteen different antibiotics; that are routinely prescribed for human treatment by using disc diffusion method (Table 2). The bacterial strains were resistant to Trimethoprim-Sulphamethoxazole (member of sulfonamides group), Chloramphenicol, Ceftazidime, Ceftriaxone and Cefoperazone. For the Quinolones/Fluoroquinolones group, strains were resistant, except for Ciprofloxacin

Table (2) 16S rRNA product Size.

Strain	Size of 16s rRNA (bp)
P. mirabilis 2	1063
P. mirabilis 10	1071
P. mirabilis 16	1115
P. mirabilis 23	1092

Table (3) Susceptibility of *P. mirabilis* isolates 2, 10, 16 and 23 to different antibiotics.

Antibiotics	**Result**			
	P. mirabilis 2	P. mirabilis 10	P. mirabilis 16	P. mirabilis 23
Amoxicillin-Clavulanate (2:1)	**R**	**R**	**R**	**R**
Azithromycin	**S**	**R**	**R**	**R**
Ceftazidime	**R**	**R**	**R**	**M**
Trimethoprim - Sulphamethoxazole	**R**	**R**	**R**	**R**
Norfloxacin	**R**	**R**	**R**	**R**
Ceftriaxone	**R**	**R**	**S**	**R**
Ciprofloxacin	**M**	**R**	**R**	**M**
Cefoperazone	**R**	**R**	**R**	**R**
Imipenem	**S**	**S**	**S**	**S**
Meropenem	**S**	**S**	**S**	**S**
Amikacin	**S**	**S**	**S**	**R**
Tazobactam	**M**	**M**	**R**	**R**
Ampicillin Sulbactam	**R**	**R**	**M**	**R**
Chloramphenicol	**R**	**R**	**R**	**R**

N: total number of patients; N^+: total number of patients with *P. mirabilis* infection; *: Statistical significance, NS: non- Statisticant.

According to CLSI standards (2012); R: Resistant; S: Sensitive; M:Intermediate sensitivity

that showed intermediate effect against *P. mirabilis* 2 and 23. Most of the isolates were resistant to Amoxicillin-Clavulanate and Ampicillin – Sulbactam, which belongs to penicillin group –except *P. mirabilis* 16 that demonstrated an intermediate effect. All strains are either resistant or have intermediate susceptibility to Tazobactam and firmly resistant to azithromycin except *P. mirabilis* 2 that showed sensitivity to this antibiotic. Additionally, strains were susceptible to Imipenem and Meropenem that belong to Carbapenems

16s RNA Polymerase Chain Reaction: The DNA encode for the 16S rRNA genes of the four seemingly different isolates were sequenced and compared with the available database using BLAST search engine. The sizes of the 16S rRNA gene products were 1063, 1071, 1092, and 1115 bp (Table 3). The sequencing data were used to construct phylogeny tree of the four distinctive strains through the online software Phylogeny.fr (Fig. 9). Count of 100 query sequences were available for comparison, *isolate 10* matched with ≥ 98.13 similarity to parital sequence of strains with accession no. MK434926.1, MN340241.1 and MK954135.1. and *P. mirabilis 16* with matching with max. similarity equal to 97.07% to 16s rRNA partial sequence of strains with accession no MH985199.1. *P. mirabilis* 2 are matching to other 5 *P. mirabilis* strains with similarity ≥ 98.04% *and mirabilis* 23 is maximally similar with 96.25% to other query sequensces

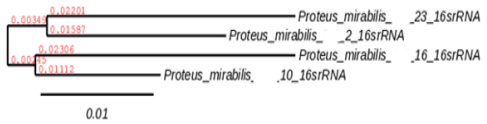

Figure (5) phylogram for four diverse strains of P. mirabilis

DISCUSSION:

The inflammatory bowel diseases (IBD) known as inflammation anywhere the gastrointestinal system, include Crohn's diseases and ulcerative colitis. The etiologic is attributed to immune dysfunctional trigger dysbiosis. Not only the microbial factors trigger IBD but also several considerable risk factors are associated, recent literature suggested the correlation between P. mirabilis and other gastric disorders **[2, 27]**. Among our

clinical survey; 68 patients diagnosed with gastric disorders, 41.18% of them were positive P. mirabilis. Ample studies correlate between gastrointestinal diseases to other factors as smoking habit and gender in which women were more positive with gastrointestinal diseases than men and this may due to gender-related biological and psychosocial difference **[27, 41-43]**. Also, our study demonstrated the high prevalence in women, however, a non-significant difference was observed among *P. mirabilis* infection with respect to gender and smoking otherwise the high-risk factor equals 1.078 and 1.154 for $p < 0.05$, respectively. In our study, 90.91% (10/11) of *P. mirabilis* male patient were smokers, our findings were in accord with similar results published by **[26, 27]**. They found a relationship between gastric inflammation and prevalence of *P. mirabilis* and the incidence was increased in smokers compared to non-smokers $\rho = 0.037$. Also, we found that, the gastroitestinal disease is more frequent in young and older age as agreed with previously published clinical surveys **[43, 44]**. Colitis were found in 73.33% of patients with high-risk factor equal to 5.8 ($p < 0.05$). Our results are in agreement with an old Russian study that concluded the existence of a correlation of *P. mirabilis* and ulcerative colitis **[24]** and in line with the recent in vivo trial that carried out in TRUC mice. The metagenomic analysis of the fecal of TRUC mice proved the prevalence of *P. mirabilis* in synery with other pathogens associated with ulcerative colitis. The compatibility between in-vitro and in-vivo antibiotic susceptibility trials emphasized the impact of the synergistic relation with *Klebsiella pneumonia* in inflammation etiology whereas, being ameliorated after the admission of susceptible antibiotics and decrease colonization of both strains **[28]**. Despite the fact that previous studies demonstrated the etiological role of *P. mirabilis* in diarrhea **[45, 46]**, our results showed no significant relationship. In addition, we did not see any significant relation between *P. mirabilis* infections and gastritis or ulcer diagnosis even though that was with risk factor equal 1.957 and 1.296 at $p < 0.05$, respectively. These results are in line with the previously reported results that showed correlation between *P. mirabilis* infection and Crohn's disease **[23, 47]**. *P. mirabilis* isolates were subjected to SDS-PAGE fingerprint by banding pattern analysis, there were four distinctive strains among the twenty-eight isolates **[48- 50]** who stated that the fractional power provides a characteristic comparative protein profiles. The phylogeny and taxonomical positions were confirmed upon 16s rRNA as a standard conservative genetic marker the divergence among the same species with <1% divergence **[51,52]**.

According to **[53-55]** our results also demonstrated that most of *P. mirabilis* isolates were resistant to a wide range of antibiotic classes include; β-lactams (penicillin and cephalosporin), fluoroquinolones, sulfonamides, and chloramphenicol. The potential possibility may be associated with the common use of these antibiotics for treatment and acquisition of resistant genes **[56-59]** were addressed that piperacillin /tazobactam is a potential therapy for extended-spectrum β-lactamase (ESBL) infections, it wasn't effective in our in vitro antimicrobial test. On the contrary, all of the isolates were susceptible to Carbapenems (Imipenem and Meropenem) as previously reported **[60, 61]**.

3. Results and Discussion

P. mirabilis has a high-risk factor to other Crohn's diseases with a significant etiological role of colitis. *P. mirabilis* infection within smoking increases the risk of pathogenicity. Only four distinctive strains have been isolated from the gastrointestinal disorder with 16s rRNA high similarity to each other. The strains had a high frequency of resistance to Amoxicillin-Clavulanate, Trimethoprim-Sulphamethoxazole, Norfloxacin, Chloramphenicol, and Cefoperazone.

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