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Isolation and Identification of Pathogenic Yersinia Enterocolotica Using 16 SrRNA from Children in AboRish Hospital, Egypt

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Abstract

Foodborne diseases are a widespread and growing public health concern in developed and developing countries. Isolation of *Yersinia enterocolitica* is include enrichment followed by streaking on selective agar such as cefsulodin, irgasan and novobiocin medium (CIN) Antimicrobial susceptibility determination of *Yersinia enterocolitica* strains was performed according to the NCCLS Standards. The use of PCR has shown to be a sufficient standard of specific identification and is not time consuming. DNA Sequencing of 16S rRNA gene was conducted in both directions and a consensus sequence of 1476 bp was used for nucleotide (nt.) analysis. The original sequences were trimmed to remove ambiguous nt. sequences usually exist in the beginning of the sequencing reaction. Partial DNA sequences were submitted to GenBank database and obtained accession numbers; MK168055 and MK168056 for Y.Ent-1/EGY018 and Y.Ent-2/EGY018 strains, respectively. Identification of homologies between nucleotide sequence of the studied Yersinia Spp. isolates and others published in GenBank was done using BLAST 2.2 search program (National Center for Biotechnology Information "NCBI" http://www.ncbi.nlm.nih.gov/). Both Y.Ent-1-EGY2018 and Y.Ent-2-EGY2018 strains revealed high similarity (96.6%) between each other. Sequence analysis of Y.Ent-1- EGY2018 and Y.Ent-2-EGY2018 showed 96.3-97.6% and 97.1-98.5% identity (respectively) when compared to other available *Yersinia enterocolitica* partial sequences of 16S rRNA gene published in GenBank. Phylogenic analysis of 16S rRNA gene partial sequence showed that Y.Ent-2-EGY2018 strain was isolating in same cluster of *Yersinia enterocolitica* but exists in different branch. However, Y.Ent-2-EGY2018 strain is present alone in a separate cluster.

Keywords: *Yersinia enterocolitica*; 16S rRNA; Phylogenic tree

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Introduction

Foodborne diseases are a widespread and growing public health concern in developed and developing countries [1]. *Yersinia enterocolitica* to the genus *Yersinia*, family *Enterobacteriaceae* of the 12 species that comprise the genus, 3 are important in human pathogenicity, namely, *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Y. enterocolitica. Yersinia pestis* is the causative agent of the bubonic plague, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are intestinal pathogens. *Yersinia enterocolitica* are widely distributed throughout the environment and have been isolated from raw milk, sewage contaminated water, soil, seafood, humans, and many warm-blooded animals, such as poultry and, most important, pigs. The serotypes 0:3, 0:5, 27, 0:8, and 0:9 are the most frequent causative agents of human illness [2]. Yersiniosis is a rare disease in Muslim countries due to the scarcity of pork consumption. The incidence of Yersiniosis is reported to be 10%–30% in European countries and 0.06%-2% in Muslim ones [3,4]. *Y. enterocolitica*, a classical enteric pathogen, causes human and animals infections whose symptoms include diarrhea, terminal ileitis, intestinal intussusception, mesenteric lymphadenitis, arthritis, and septicemia [5]. As a result of the host's immune response, *Y. enterocolitica* may also induce secondary, post infectious squeal such as acute and chronic arthritis, erythema nodosum, and septicemia [6-8]. *Y. enterocolitica* is responsible for 50% of all the clinical sepsis episodes that occurs as a result of transfusion of contaminated red blood cells [7]. In 2007, there were 8,792 reported cases of human yersiniosis in the European Union, making Yersinia the third most important zoonotic agent implicated in human enteritis, in terms of the number of cases, after Campylobacter and Salmonella [10]. Isolation of *Y. enterocolitica* is include enrichment followed by streaking on selective agar such as cefsulodin, irgasan and novobiocin medium (CIN) [11,12]. Single colonies of bacterial strains were picked and further grown and sub-cultured several times to obtain a pure culture. Biochemical characterizations of the strains were performed using API 20E system [13]. Traditionally, bacteria were classified according to similarities and differences in phenotypes, such as

morphology and biochemical reactions. The API 20E strip test is the standard for identifying Enterobacteriaceae; however, this test has limitations in identifying Yersinia species. Several Yersinia species can be identified using API 20E strip test where the accuracy is influenced by passage number, culture conditions, and instability of some biochemical reactions [14]. Pathogenic strains of *Y. enterocolitica* are transmitted to man mainly from contaminated water or food (meat, milk or vegetables), more specifically raw or undercooked pork, and they may cause various infectious diseases (enteritis, enterocolitis, mesenteric lymphadenitis) [15]. Antimicrobial susceptibility determination of *Y. enterocolitica* strains was performed according to the [16]. The great majorities of the gastrointestinal infections is self-limiting and confined to the gut and do not merit antimicrobial therapy in an immunocompetent host. However, antimicrobial therapy is warranted to treat enterocolitis in compromised hosts and in patients with septicemia or invasive infection, in which the mortality can be as high as 50%. Despite antibiotic susceptibility patterns varying among serogroups, the organism is usually susceptible in vitro to aminoglycosides, cotrimoxazole, chloramphenicol, tetracycline, third generation cephalosporins and fluoroquinolones but is resistant to penicillin, ampicillin and first generation cephalosporins. The intrinsic resistance to these beta-lactam antibiotics is due to the production of two chromosomally encoded beta-lactamase genes, blaA and blaB, encoding for one class A enzyme showing constitutive expression and one inducible class C enzyme (AmpC-type), respectively [17,18]. In this study, we evaluated the performance of the VITEK2 system for identification and antimicrobial susceptibility testing of routine unselected *Enterobacteriaceae* clinical isolates, which were obtained consecutively in our laboratory. Molecular methods, including TaqMan assays or antigen detection tests, offer more rapid and sensitive detection than conventional culture methods. Since the 1980s, the ribosomal RNA gene has been used for phylogenetic studies, and ribosomal RNA-based approaches have been increasingly applied to bacterial classification and identification, especially using the 16S rRNA. The 16S rRNA gene is generally accepted as the best molecular sequence to use for identification because it is functionally constant and shows a mosaic of structure having conserved and variable regions and exists in all organisms; and its length is easily sequenced [19]. The diversity in 24 species is close to or over the threshold of the 16S rRNA gene-based operational definition of a species (1% to 1.3% diversity) [20], so these species maybe misclassified into a new species if a different copy is used for identification. Recently, 2,013 genomes were analyzed and22.5% were found divergence at over 1% in the 16S rRNA gene copies [21]. In addition, the 16S rRNA gene shows high similarity comparing some different species where identical sequences were found even [22]. This limits the use of the 16S rRNA gene in identifying bacterial species. Therefore, sequencing the 16S rRNA gene from multiple operons from isolates is recommended to achieve significant phylogenetic information for species identification [23]. Sequencing of genes coding for the small subunit ribosomal 16S rRNA is an important tool for the identification of bacteria based on their phylogenetic relationships. It is one of the primary tests used in the classification of novel species. Stackebrandt and Goebel (1994) introduced a 16S rRNA gene sequence similarity value of 97% to indicate strains belonging to the same species. This threshold value has been suggested to be corrected to98.7-99.0% [24,25]. However, this method cannot be used alone to differentiate two species. Some species share a value of more than 99% even though they represent different species [26].

Material and Methods

The study was conducted during the period from January-2018 to January-2019 on 300 patients, from Abu-Resh Children's Hospital. (Cairo University), Egypt. The patients were suffering from recurrent Gastroenteritis included (diarrhea, vomiting, fever). A Stool samples (300 samples) following infection control procedures were collected after taking the patient's consent. All patients are with the same age range (6 Months -14 Years old).

All participants were subjected to:

- Detailed history: the duration of recurrent Gastroenteritis, if under any treatment, especially antibiotics, its duration, dose and type.
- Participants who were on antibiotics were asked to come one week after the last dose.

Fecal samples were collected during the active phase of infection (under sterile conditions) in sterile containers and then transferred to the bacterial laboratory within half an hour.

The fecal sample were inoculated into PBS containing 0.15% bile salts and 1% sorbitol and incubated at 4oC for 3 weeks. *Y. enterocolitica* colonies are characterized by their unique bull's-eye morphology on the CIN agar (1.5 mm diameter, deep red/purple center with a sharp edge surrounded by a translucent border). Therefore, only colonies with suspected morphologies were selected and tested by gram staining**.** The colonies were then grown on CIN agar, and the cells with a typical dark red center known as the "red bull's eye" were transferred to blood agar and used for biochemical testing and PCR. For confirmation of genus Yersinia Gram's staining, motility test and various biochemical reactions like oxidase test, urease test, citrate test, acid and alkali reaction on Kligle rIron agar (KIA) slants were performed**.** One bacterial colony was suspended in 10 ml PBS (phosphate- buffered saline) and adjusted to McFarland standard 5.0. The API 20E strips were inoculated following the manufacturer's instructions. Because of the temperaturedependent Voges–Proskauer test, the API 20E system was incubated for 18-20 h at 25℃. All isolates were Identified & Susceptibility using the database provided by the manufacturer the Vitike 2 compact system (bioMerieux).

The PCR primers used in this study of *Yersinia* and plasmid of *yersinia* were prepared by Sigma Company (Table 1).

The first set of the primer was used for detection and identification of the isolated ***Yersinia enterocolitica*** and the other for plasmid.

DNA extraction from isolates using Qiagen extraction kit as describe Tissue supernatant (samples) was placed by 50- 200 µl (media, culture cells) in eppendorf tube.

- Equal volume from the lysate (50-200 µl) was added, addition of 20-50 µl of proteinase K, then incubation at 56°C for 20-30 min.

Table 1: Primers used for detection of the 16 S-rRNA genes of *Yersinia enterocolitica.*

Primer pairs	Sequence (5′→3′)	Amplicon size (bp)	Reference
16S-rRNA		1,425 bp	Anna Murros (2017) [27]
F	CTGGCTCAGGAYGAACGCTG		
R	AAGGAGGTGATCCAGCCGCA		

- The solution was added to the column and centrifuged at 8000 rpm for 1 min. then the filtrate was discarded.
- The sediment was washed using AW1 buffer (200 µl), the column was centrifuged at 8000 rpm / 1 min, and the filtrate was discarded.
- Washing was applied by using the AW2 buffer (200 µl), the column was centrifuged at 8000 rpm / 1 min. and the filtrate was discarded.
- The column was placed in a new clean tube then, 25-50 µl from the Elution buffer was added, centrifuged at 8000 rpm / 1 min. Then the column was discarded. The filtrate was put in the clean tube contains the pure genomic DNA.

Method by manufacturer manual of Qiagen, USA, as following:

Method for Extraction Plasmid

- Pick a single colony from a freshly streaked bacterial plate and use it to inoculate LB (+antibiotics). And then grow at 37 for 12 ~ 16 hrs. with vigorous shaking (OD600 = 1.0 ~ 1.5).
- Harvest 3-5 ml of bacteria culture by centrifugation at 13,000 rpm for 30 sec at RT and discard supernatant. Note: Drain tubes on a paper towel to remove excess media.
- Resuspend pelleted bacterial cell thoroughly in 250 µl of resuspension buffer by vortexing until no clumps remain.

Note: Ensure that RNase A solution has been added to resuspension buffer. It is essential to completely resuspend the cell pellet. It may affect the lysis efficiency

- Add 250 µl of Lysis Buffer to resuspended cells and mix by inverting the tube 10 times. DO NOT VORTEX and incubate for 3 min at RT.

Note: The optimal lysis time allows maximum release of plasmid DNA without release of chromosomal DNA, while minimizing the exposure of the plasmid to denaturing conditions. Long exposure to alkaline condition may cause the plasmid to become irreversibly denatured. It is important to proceed to next step immediately after the lysate becomes clear without any cloudy clumps. Do not vortex, it may cause shearing of genomic DNA.

Note: If the Lysis buffer becomes too cold, SDS precipitation may occur, leading to poor cell lysis. If a precipitate has formed, warm the Lysis buffer to 37°C with gentle shaking.

- Add 350 µl of Neutralization Buffer and gently mix by inverting the tube 10 times then incubate the tube in ice for 5 min.

Note: After addition of Neutralization Buffer, the solution should become cloudy and a fluffy white form. Incubation on ice may help precipitating the denatured cell components more efficiently.

- The precipitated material contains genomic DNA, protein, cell debris, and SDS.

Note: If LysisViewer reagent has been used, the suspension should be mixed until all trace of pink has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

- Centrifuge at 13,000 rpm for 10 min at 4°C. While waiting for the centrifugation, insert a column into collection tube.
- After centrifugation, transfer supernatant promptly into the column.

Table 2: PCR protocol for amplification conditions of 16S rRNA gene.

Steps	Temp. (°C)	Time	No. of cycles
Initial Denaturation	98	3min	One cycle
Denaturation	93	60 sec	30 cycles
Annealing	54	120 sec	30 cycles
Extension	72	150 sec	30 cycles
Final extension	72	8 min	One cycle

Note: Cell debris, protein and genomic DNA will form a compact white pellet in the tube. Do not transfer with white pellet.

- Centrifuge at 13,000 rpm for 1 min. Remove the column from collection tube, discard filtrate in collection tube. And then place the spin column back in the same collection tube.
- (Optional) Add 500 µl of Washing Buffer A and centrifuge at 13,000rpm for 1 min remove the column from collection tube, discard filtrate in collection tube. And then place the spin column back in the same collection tube.

Note: This step is necessary to remove trace nuclease activity. endA+ strains, such as BL21, HB101, JM series, or any wild-type strains, have high level of nuclease activity that can degrade plasmids. But endA- strains, such as DH5α, XL1-blue and etc., do not require this additional washing step.

- Add 700 µl of Washing Buffer B and centrifuge at 13,000rpm for 1 min., discard filtrate in collection tube and then place the spin column back in the same collection tube.
- Centrifuge to dry the filter membrane.
- Transfer the spin column to a new 1.5 ml or 2 ml micro centrifuge tube, and add 50 µl elution buffers or distill water to the upper reservoir of the column and let to stand for 1 min at room temperature. Centrifuge for 13,000 rpm for 1 min (Table 2).

Identification of the PCR Products by Agarose Gel Electrophoresis

PCR products were analyzed for the presence of specific fragments of the expected length in a 1.5% agarose gel electrophoresis stained with Ethidium bromide.

Powdered electrophoresis agarose was prepared of 1X electrophoresis buffer in a flask with a loose fitting cap to reach the required concentration (1.5%). The buffer should not occupy more than 50% of the flask. The mixture was heated in a microwave with periodical agitation until the agarose dissolves. Solution was cooled to 60°C, and then ethidium bromide was added from a stock solution of 1 µl /gel and mixed thoroughly. The combs were placed 0.5-1.0 mm above the plate so that a complete well was formed when the agarose was added. The gel mixture was then poured directly into movable casting apparatus. The gel thickness was 3-5 mm with no air bubbles under or between the teeth of the comb. After the gel was completely set (30-45 minutes at room temperature), the comb was carefully removed. Electrophoresis buffer was added to cover the gel to a depth of about one mm. The PCR product (5 µl) and molecular weight maker were loaded. The PCR products were loaded into the slots of the submerged gel using micropipette. The lid of the gel tank was closed and attached to the power supply. The running parameters were 1-5 volt/cm (measured as distances between electrodes). The gel was run until the bromothymol blue and xylene cyanol had migrated the appropriate distance through the gel (2/3 of the gel length). After the electric current was turned off, the lid of the gel was removed and the gel was transferred to trans-

Table 3: Incidence of Yersinia spp. isolated from the stool culture of 300 patients.

Species	No. of isolates	% of patients (n=300)	% of isolates (n=5)	Incidence (Per 1000 patient)
Y.enterocolotica	5	1.66	100	16
Male	3	1.0	60	10
Female	2	0.66	40	6

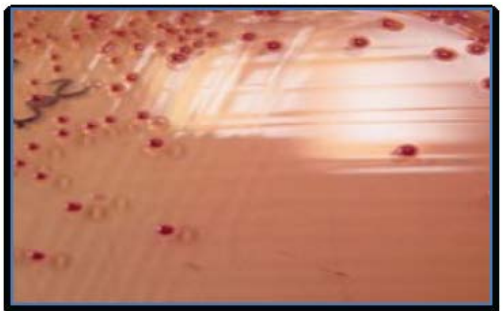

Figure 1: CIN agar.

Figure 2: MacConkey agar.

illuminator to observe the amplified DNA on the gel in comparison to molecular weight markers. The gel was photographed. We made PCR clean up to the PCR product using GeneJET PCR Purification Kit as following:

- Add a 45 µl of Binding Buffer to completed PCR mixture. Mix thoroughly.
- Transfer the mixture from step 1 to the GeneJET™ purification column. Centrifuge for 30-60 sec at & gt; 12000 x g.
- Add 100 µl of Wash Buffer to the GeneJET™ purification column. Centrifuge for 30-60 sec. Discard the flow through and place the purification column back into the collection tube.
- Centrifuge the empty GeneJET™ purification column for an additional 1 min to completely remove any residual wash buffer.
- Transfer the GeneJET™ purification column to a clean 1.5 ml micro centrifuge tube. Add 25 µl of Elution Buffer to the center of the GeneJET™ purification column membrane and centrifuge for 1 min.
- Discard the GeneJET™ purification column and store the purified DNA at -20°C.

Results

The cold enrichment culture was done for all 300 patients studied. The total number of Yersinia isolates from the stool culture of all patients studied was 5 (1.66%); The 5 isolates of *Y. enterocolitica* were

Table 4: Age and sex Distribution of 300 Patient of children with acute diarrhea.

Age	Male	Female	Total	%
< 1	20	15	35	11.7
1-3	100	60	160	53.3
4-7	50	30	80	26.7
8-14	20	5	25	8.3
Total	190	110	300	100

Table 5: Antibiogram of *Y. enterocolitica*isolates.

Susceptibility Information	Analysis time	17:97 hrs		Status	Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL			Meropenem	<=0.25	S
Ampicillin	>=32	R	Amikacin	<=2	S
Ampicillin/ Sulbactam	16	I	Gentamicin	<=1	S
Piperacillin/ Tazobactam	TRM		Tobramycin	<=1	S
Cefazolin	>=64	R	Ciprofloxacin	<=0.25	S
Cefoxitin	32	R	Levofloxacin	<=0.12	S
Ceftazidime	<=1	S	Nitrofurantion	32	S
Ceftriaxone	<=1	S	Trimethoprim/ Sulfamethoxazole	<=20	S
Cefepime	<=1	S			

Whereas: += Deduced; *=AES modified; **=User modified.

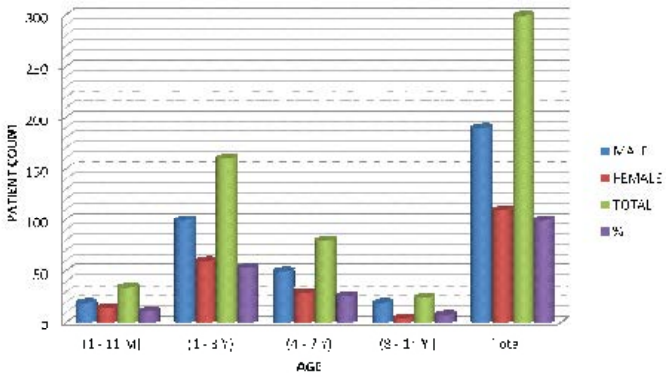

Figure 3: Age & sex Distribution of 300 Patient of children with acute diarrhea.

recovered from children 3 male 190 (63.33%) aged (1,2, and 5) years old, while the 2 female age 110 (36.66%) (1,5) years old (Table 3) (Figure 3).

Typical dark red colonies surrounded by transparent border; usually flat with smooth border and entire edge giving a characteristic "Bulls-Eye" appearance, were observed after 72 hrs. of incubation on CIN agar (Figure 1) in case of positive samples, whereas in MacConkey agar, the colonies were pale pink (Figure 2). Gram staining revealed gram negative coccobacillary morphology.

Five bull's-eye colonies were identified on CIN media and therefore were suspected to be *Y. enterocolitica.* Gram staining showed that all suspected colonies are negative rods. Finally, API test had confirmed isolates as *Y. enterocolitica* by Vitek2 Compact system (BioMérieux).

The isolate recovered under present study showed high sensitivity to Ceftazidime, Ceftriaxone, cefepime, amikacin, ciprofloxacin, Gentamicin, Tobaramycin, Meropenem, Levofloxacin, Nitrofurantion, and Trimethoprin/Sulfamethoxazole. Whereas moderate sensitivity towards Ampicillin/Sulbatam (Table 5). The isolates were resistant against Ampilcillin, Cefoxitin and Cefazolin (Table 5).

In our we made sequencing to the PCR product on GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers. DNA Sequencing of 16S rRNA gene was conducted in both directions and a consensus sequence of 1476 bp was used for nucleotide (nt.) analysis. To detect the gene (16SrRNA,) using the PCR methods, specific sequences of genes were amplified and individual amplified fragments were detected by agarose gel electrophoresis (Figure 4).

Where: Lane 1: 100 bp plus DNA, Gene Ruler™, Fermentas

Lane 2: Isolate 1

Lane 3: Isolate 2

Lane 4: Control Negative Lane 5: Control Positive

Partial DNA sequences were submitted to GenBank database and

Figure 4: Electrophoretic pattern of PCR product of 16S rRNA gene for Yersinia.

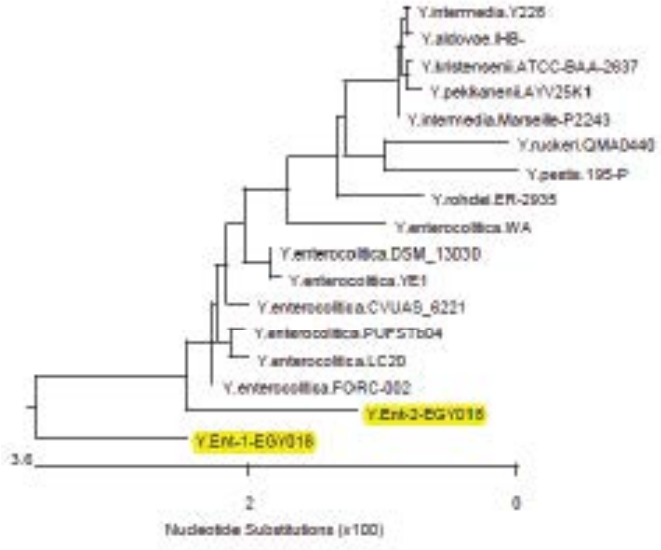

Figure 5: Phylogenic tree analysis of 16S rRNA gene partial sequence.

obtained accession numbers; MK168055 and MK168056 for Y.Ent-1/EGY018 and Y.Ent-2/EGY018 strains, respectively. Phylogenic analysis of 16S rRNA gene partial sequence showed that Y.Ent-2-EGY2018 strain was isolating in same cluster of *Yersinia enterocolitica* but exists in different branch. However, Y.Ent-2-EGY2018 strain is present alone in a separate cluster (Figure 5).

Both Y.Ent-1-EGY2018 and Y.Ent-2-EGY2018 strains revealed high similarity (96.6%) between each other. Sequence analysis of Y.Ent-1-EGY2018 and Y.Ent-2-EGY2018 showed 96.3-97.6% and 97.1-98.5% identity (respectively) when compared to other available *Yersinia enterocolitica* partial sequences of 16S rRNA gene published in GenBank. On the other hand, Both Y.Ent-1-EGY2018 and Y.Ent-2-EGY2018 showed the highest similarity, 97.6% and 98.5%, respectively, comparing to *Y. enterocolitica* FORC-002 strain (Figure 6).

Partial 16S rRNA gene sequence alignment showed that Y. Ent-1-EGY2018 gained 46 unique nucleotide changes; while Y. Ent-2-EGY2018 showed 35 unique nucleotide changes comparing to the available published *Yersinia spp.* sequences. Both Y. Ent-1-EGY2018 and Y. Ent-2-EGY2018 strains shared 15 unique nucleotide changes (Figure 5). Finally, we made sequencing to the PCR product on GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers. Only by combining the traditional Sanger technology with the new 454 technologies, can genomes now be sequenced and analyzed in half the usual project time, with a considerable reduction in the number of coatings and gaps. In addition, considerable cost advantages now make genome sequencing with the 454 technology accessible to the research community. DNA Sequencing of 16S rRNA gene was conducted in both directions and a consensus sequence of 1476 bp was used for nucleotide (nt.) analysis. The original sequences were trimmed to remove ambiguous nt. sequences usually exist in the beginning of the sequencing reaction. Partial DNA sequences were submitted to GenBank database and obtained accession numbers; MK168055 and MK168056 for Y. Ent-1/EGY018 and Y.Ent-2/EGY018 strains, respectively. Identification of homologies between nucleotide sequence of the studied Yersinia Spp. isolates and others published in GenBank was done using BLAST 2.2 search program (NCBI). Comparisons of the obtained nucleotide sequence with other Yersinia sequences that published in GenBank were done using the BioEdit sequence alignment editor [28] and MegAlign, DNASTAR, Lasergene®, Version 7.1.0, USA. The phylogenetic trees were constructed using MegAlign for tree reconstruction of sequences by Neighbor-joining method based on ClustalW [29]. Sequence divergence and identity percentages were calculated by MegAlign. Sequences were analyzed and aligned by Clustal method using the program DNA star (Lasergene, Wisconsin, USA).

Discussion

In this work, the biochemical tests were used to determine the assumed *Yersinia enterocolitca* isolates. To confirm the identification results of potentially zoonotic isolates we used VITEK® 2 Compact, and PCR. Antimicrobial therapy is not usually recommended for treating enterocolitis in immune competent hosts since most of the gastrointestinal infections are self-limiting. However, immunocompromised patients with invasive infection, who are at increased risk for developing bacteremia or even septicemia, need special attention and antibiotic treatment since the mortality rate in these cases, can be as high as 50% [30]. According to the common profile of susceptibility among *Y. enterocolitica* strains (see section on "Antimicrobial Susceptibility"), the initial recommendations for

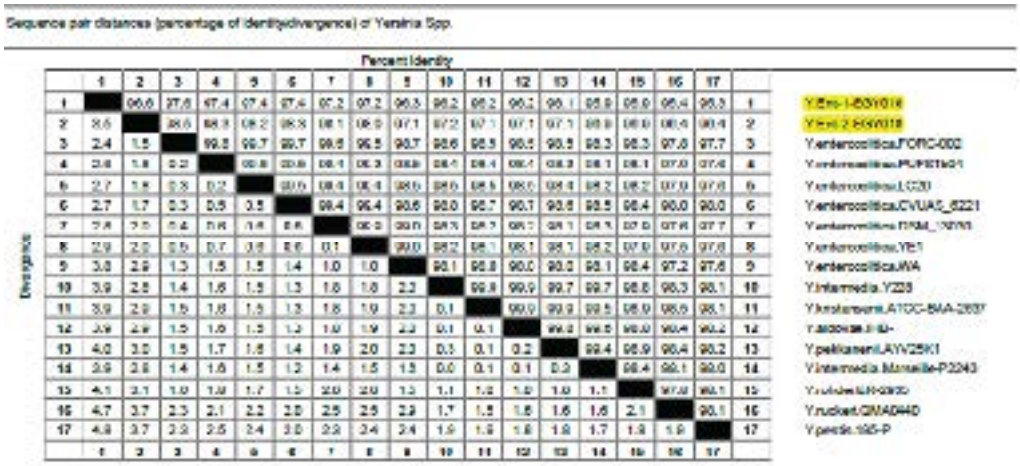

Figure 6: Sequence distance (percentage of identity divergence) of Yersinia Spp.

antimicrobial chemotherapy from public institutions, such as the WHO, included tetracycline, chloramphenicol, gentamicin, and cotrimoxazole [31]. Although several antimicrobials are commonly active against *Y. enterocolitica* in vivo, like aminoglycosides, cotrimoxazole, chloramphenicol, tetracycline, third generation cephalosporins and fluoroquinolones, in vitro susceptibility to antimicrobials varies among bioserotypes. The microorganism is usually resistant to penicillin, ampicillin and first generation cephalosporins [32]. The resistance to the beta-lactam antibiotics is mediated by the chromosomal genes blaA and blaB, which encoded constitutively, produced beta- lactamases [33]. The most effective drugs (full sensitivity) in treating yersiniosis were gentamicin, cefotaxime, chloramphenicol and tetracycline. This result is in agreement with that of Tzelepi E, et al. (1999) [34]. All the strains of *Y. enterocolitica* isolates samples were sensitive to amikacin and gentamycin (100 %). Susceptibility of the isolates to amikacin and gentamycin were in accordance with earlier studies [35-38]. In our study *Y. enterocolitica* intrinsic resistance to ampicillin, cefazolin, cefoxitin and this is consistent with other studies assigned by the Clinical & Laboratory Standards Institute and EUCAST [39,40]. The recent development of sensitive, specific PCR assays for the detection of Yersinia organisms has greatly improved the ability to study this organism in fresh and fixed samples. Because *Y. enterocolitica* is a common foodborne pathogen and is implicated in such a wide range of gastrointestinal diseases, the development of a PCR assay that could be used to assign *Y. enterocolitica* positive specimens to a particular bio group has significant implications for clinical diagnosis, microbiological research, and epidemiological studies [41,42]. Compared to bacteria identification methods using phenotype, the approach based on genotype stands out for its consistency. One desirable candidate is the 16S rRNA gene, highly conserved and seldom variable within species, and is becoming an important technique for phylogeny research and species classification which can be made up through the method based on 16S rRNA gene [43]. However, the similarity of 16S rRNA gene sequence between species is as high as 96.9%-99.8%; it is easy therefore to misclassify species of high homology [44]. A number of DNA-based methods for identification of zoonotic *Y. enterocolitica* strains such as PCR, and DNA sequencing was introduced [45]. Three of them have used the 16S rDNA-specific PCR designed by Neubauer H, et al. (2000) for accurate and rapid species confirmation of *Y. enterocolitica* [46,47]. In other PCR techniques identification of *Y. enterocolitica* were based on detection of particular species-specific virulence genes [48-51]. The 16S rDNA sequence analysis is still the gold standard in microbial identification. In this paper 16S rDNA sequence analysis was used to reveal the species membership of two children isolates. In our study Phylogenic analysis of 16S rRNA gene partial sequence showed that Y.Ent-2-EGY2018 strain was isolating in same cluster of *Yersinia enterocolitica* but exists in different branch. However, Y.Ent-2-EGY2018 strain is present alone in a separate cluster (Figure 4). Both Y.Ent-1-EGY2018 and Y.Ent-2-EGY2018 strains revealed high similarity (96.6%) between each other. Sequence analysis of Y.Ent-1-EGY2018 and Y.Ent-2-EGY2018 showed 96.3-97.6% and 97.1-98.5% identity (respectively) when compared to other available *Yersinia enterocolitica* partial sequences of 16S rRNA gene published in GenBank. On the other hand, Both Y.Ent-1-EGY2018 and Y.Ent-2-EGY2018 showed the highest similarity, 97.6% and 98.5%, respectively, comparing to *Y. enterocolitica* FORC-002 strain (Figure 5).

Partial 16S rRNA gene sequence alignment showed that Y.Ent-1-EGY2018 gained 46 unique nucleotide changes; while Y.Ent-2-EGY2018 showed 35 unique nucleotide changes comparing to the available published *Yersinia spp.* sequences. Both Y.Ent-1-EGY2018 and Y.Ent-2-EGY2018 strains shared 15 unique nucleotide changes (Figure 5).

Finally we made sequencing to the PCR product on GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers. Only by combining the traditional Sanger technology with the new 454 technology, can genomes now be sequenced and analyzed in half the usual project time, with a considerable reduction in the number of coatings and gaps. In addition, considerable cost advantages now make genome sequencing with the 454 technology accessible to the research community. DNA Sequencing of 16S rRNA gene was conducted in both directions and a consensus sequence of 1476 bp was used for nucleotide (nt.) analysis. The original sequences were trimmed to remove ambiguous nt. sequences usually exist in the beginning of the sequencing reaction. Partial DNA sequences were submitted to GenBank database and obtained accession numbers; MK168055 and MK168056 for Y.Ent-1/EGY018 and Y.Ent-2/EGY018 strains, respectively. Identification of homologies between nucleotide sequence of the studied Yersinia Spp. isolates and others published in GenBank was done using BLAST 2.2 search program (National Center for Biotechnology Information "NCBI" http://www.ncbi.nlm.nih.gov/). Comparisons of the obtained nucleotide sequence with other Yersinia sequences that published in GenBank were done using the BioEdit

sequence alignment editor [28] and MegAlign, DNASTAR, Lasergene®, Version 7.1.0, USA. The phylogenetic trees were constructed using MegAlign for tree reconstruction of sequences by Neighbor-joining method based on ClustalW [29]. Sequence divergence and identity percentages were calculated by MegAlign. Sequences were analyzed and aligned by Clustal method using the program DNA star (Lasergene, Wisconsin, USA).

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