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Diagnosis of Achromobacter xylosoxidans and Klebseilla oxytoca as Etiological Agents of Peptic Ulcers

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Abstract: Helicobacter pylori is the pathogen only known that inhabits the gastric mucosa of almost half of the world's population, and the bacterium is associated with higher incidence of peptic ulcer worldwide. The present study was aimed to seek for diagnosis of H. pylori as etiological agents of peptic ulcer in Iraqi patients on second line therapy and suffering from severe ulcer reinfection after a period of time. Sixty-five endoscopic gastric biopsy specimens were obtained from patients of both genders and in age around 45-60 years. For primary isolation, 26 (40%) out of all corpus and antrum screened biopsies were positive on supplemented Columbia agar. Culture isolates showed heteroresistance pattern to antibiotics used in triple therapy regimen for eradication of H. pylori infections, in that high percentage of resistance to tetracycline and metronidazole (100%) was recorded, while most of isolates were sensitive and in various degrees (27%, 12.5%, and 25%) to amoxicillin, clarithromycin, and levofloxacin respectively, on the other hand one isolate exhibited absolute resistance to all of the tested antibiotics. The molecular detection of 16S rRNA (109bp) and ureA (411bp) genes specific for H. pylori were not detected by PCR amplification. Two isolates which showed significant similarities to H. pylori throughout the morphological and cultural examination, were selected and subjected to molecular analysis via 16S DNA sequencing. GenBank BLAST analysis was showed that the isolates were non-H. pylori isolates, rather, one was identified as Achromobacter xylosoxidans and the other Klebsiella oxytoca, with 98% and 97% identical gene sequences respectively. .

Keywords: Helicobacter pylori, Colombia agar medium, Standard triple therapy, Proteobacteria, Klebsiella oxytoca, Achromobacter xylosoxidans

Introduction

Helicobacter pylori is spiral shaped, Gram-negative microaerophilic microorganism, naturally found in more than 50% of the world's human population who are asymptomatic and healthy (1), with fewer than 15% of the carriers developing disease like gastroduodenal diseases such as duodenal and gastric ulcers. Furthermore, the bacterium could be the trigger of various malignant diseases of the stomach (2). Quick evolution of multidrug resistant isolates and no vaccine until today lead to global spread of H. pylori and cause worrying health disorders worldwide especially in developing countries (3). In respect of isolation and diagnosis of H. there are many noninvasive diagnosis methods, such as the antibody-based tests like stool antigen test, serological diagnosis and urea breath test (4). And several invasive (direct) tests, which is routinely most followed once. These tasting depends on mucosal endoscopic biopsies , such as rapid urea test, histopathological sections examination and laboratory cultivation. Cultivation based on using of specific and highly selective bacteriological culture media (5). The endoscopic gastric biopsies cultivation must achieved in 2-8% O2, 10% CO2, and incubation for more than 3 days. Cultural strains are identified, through means such as, Gram staining, catalase, oxidase and urease as one of classical, effective and sensitive diagnosis procedure for H. pylori (6). Although the culture method is a "gold standard" for diagnosing many infectious diseases, it is not easy in the case of an H. pylori infections diagnosis. The invasive cultural methods sensitivity of the H. pylori isolation shows a marked variation and now decreased, owing to the small population of bacteria that colonizes the stomach, and two or three biopsy specimens from different locations are requested for accurate diagnosis (7). Moreover, the positive cultural results are not always guaranteed and easily vanished under normal atmospheric conditions (8). Therefore, a number of molecular detection procedures have been developed that have been extensively used, the PCR methods that involves several housekeeping genes like; 16S rRNA, rpoD, ureA,

ureB, and ureC to detect H. pylori in biopsy specimens, and culture isolates (9, 10). The optimal treatment for H. pylori infections has not been yet found. Clinical treatment for eradicate of H. pylori infection, routinely is combination therapy, triple or quadruple antibiotic therapy and remains the worldwide choice for eradicate H. pylori infections in patients with both gastric and duodenal ulcers (11). Triple therapy involves combination of two different antibiotics (clarithromycin or metronidazole, amoxicillin, tetracycline or rifabutin), plus proton pump inhibitors (PPI) for 7 to 14 days (12). The eradication cure rates of this dependent regimen are relatively low as 50 – 70 % (13), due to the high rates of antibiotic resistance (14). Know it was noticing the PPI based treatment for more than two weeks changes the stomach pH to more alkaline conditions, and this may cause inflammation of the stomach lining and induced glandular atrophy (15). The documented studies referred, the elimination of H. pylori encouraged some other opportunistic microorganisms to thrive, enabling more severe gastric infections and gastritis to spread to the upper compartment of the stomach, which could ultimately promote the development gastric cancer (16). Currently studies have been shed light on the impacted association of H. pylori with human stomach microbiota. It was reported abundance of non-helicobacter bacteria, particularly from Proteobacteria (to which H. pylori belongs) in gastric microbial profiles of H. pylori positive patients (17). In vivo Murine model studies supported the role of non-H. pylori, particularly proteobacterial members in the development of gastritis from peptic ulcers to gastric neoplasia (18). Such studies in positive H. pylori patient's demonstration has been showed gastric environment neutralization, and this closely correlated the alterations in the gastric microbiota and significantly increased colonization of proteobacteria (19).

The aim of this study was to seek for the prevalence of H. pylori isolates as etiological agent for peptic ulcer in patient suffering from prolonged recurrent peptic ulcer, by using the cultural and molecular detection procedures.

Materials and Methods

Specimen Collection

Sixty-five endoscopic gastric biopsy specimens were taken by specialist from corpus and antrum region of patient's stomach in age of 45 – 60, who were previously diagnosed with peptic ulcers. The study enrolled patients were attended the Gastroenterology Department/ Baghdad teaching hospital, Baghdad/ Iraq. All patients were on second line ulcer antibiotic therapy and not recovered after months of massive medication. The obtained biopsies were placed in separate tubes containing sterile phosphate buffer saline (PBS) pH 7, and transported to the laboratory within 1-2 hrs.

Cultivation of Bacteria

The biopsy specimens were gently ground and homogenized, a loop full of the homogenate mixtures were streaked on supplemented Columbia agar (Micromedia/Hungary), agar base enriched with 7% (v/v) horse blood and supplemented with Dent's supplement, which is a combination of three different antibiotics; amphotericin B 5 µg.ml.-1, trimethoprim 5 µg.ml-1, and vancomycin 10 µg.ml-1 (20). The plates were incubated in 95% humidified atmosphere with 10% CO2 at 37°C, for 6-7days. Pronounced colonies were sub-cultured on Columbia agar and brain heart infusion supplemented with 5% (v/v) sheep blood. The colonies were identified on the bases of colony morphology, Gram staining, catalase, oxidase and urease testing results.

Antibiotic susceptibility

The antibiotic susceptibility profile against routinely used antibiotics in triple therapy regimen for eradication of H. pylori, including amoxicillin 2µg (AMO,), tetracycline 30 µg (TET), metronidazole 5 µg (MET), clarithromycin 15µg (CLT) and levofloxacin 5µg (LEV), was performed using the disk diffusion method (Kirby–Bauer) on Muller Hinton (MH) agar medium (Himedia/ India). In brief; fresh overnight bacterial cultures in brain heart infusion broth were adjusted to McFarland tubes No. 0.5, (108 CFU/ml), and aliquots 100 µl of each culture was individually swabbed on the surface of MH agar, antibiotic discs were gently placed on the agar surface under strictly aseptic conditions, plates were incubated at 37°C for 48 hrs. The inhibition zone diameters around antibiotic disks were measured and interpreted as recommended by the Clinical Laboratories Standard Institute (21). Some of the isolates were not able to grow on the Muller Hinton agar medium, therefore horse blood was added to the culture medium to support growth.

PCR identification of Bacterial isolates

The DNA of the bacterial isolates that thought to be H. pylori, was extracted by using genomic DNA purification kit (Intron Biotechnology, Korea), and according to manufactures instructions. All isolates were subjected to PCR analysis to identification the housekeeping genes. The primer pair Hp1F/HP2R was used to target the 16S rRNA gene specific for H. pylori and HPU1F/HPU2R was used to target the ureA gene (Integrated DNA technologies / Canada). The universal primer 27F/1492R was used to detect the bacterial universal 16S rRNA gene of the bacterial isolates (Macrogen/Korea) as shown in table (1). The reaction programs of PCR are listed in table.2. The program was held at 4 °C, and then the PCR products were visualized by using of gel electrophoresis..

Table 1. Sequence of primers used for identification the gastritis associated bacteria

Gene	Oligonucleotide	Sequence	Reference
16S rRNA specific for *H. pylori*	Hp1F/HP 2R	5′ CTGGAGAGACTAAGCCCTCC 3′ 5′ ATTACTGACGCTGATTGTGC 3′	(22)
ureA gene	HPU 1F/HPU 2R	5′ GCCAATGGTAAATTAGTT 3′ 5′ CTCCTTAATTGTTTTTAC 3′	(23)
universal *16S rRNA* gene	27F/1492 R	5′ AGAGTTTGATCCTGGCTCAG 3′ 5′ CGGTTACCTTGTTACGACTT 3′	(24)

Table 2. Program used for amplify the 16 rRNA, ureA genes, and universal 16 rRNA

Primer	Initial denaturation	Denaturation	Annealing	Extension	Final extension
		40 cycles			
Hp1F/HP2R	95 °C (10 min)	95 °C (30 sec)	55-60°C (1 min)	72°C (1 min)	72°C (5 min)
HPU1F/HPU2R	95 °C (10 min)	95 °C (10 min)	45 °C (1 min)	72°C (1 min)	72°C (5 min)
20F/1530R	95 °C (3 min)	95 °C (45 sec)	62°C (45 sec)	72°C (1 min)	72°C (10 min)

Sequencing and Sequence Alignment

The sequence of universal 16S rRNA gene of isolates 3 and 9 was carried out by sending the PCR products of amplified 16S rRNA gene to Macrogen Company/ Korea to preform Sanger sequencing by using AB13730XL, automated DNA sequences. The result analyzed by BLAST website on NCBI.

Results

Cultivation and preliminary identification

Out of sixty-five analyzed corpus and antrum biopsies, 26 (40%) were pronounced visible colonies on supplemented Columbia agar. The gastritis associated bacterial (GAB), harvested colonies were tiny, pinpoint, transparent, convex, and circular (Fig. 1). The GAB isolates were number coded (1-26). The respective colonies were further taken for Gram staining and microscopic examination, those that stained Gram negative were selected. The shape of the bacteria appeared as long twirled, with varying lengths and seemed to be segmented, with curved rod-wing shapes, it seemed like these long bacterial cells were broken down into pieces forming wing and rod like shapes, spiral shapes were also noticed, and coccoid morphology was dominant in old cultures that were kept at 4°C for a week or more.

All GAB isolates were catalase and oxidase positive, while, only eight isolates have been positive for urease, all the urease negative isolates were eliminated from study due to mismatch with the control.

Antibiotic susceptibility

The GAB isolates antibiotic susceptibility was analyzed against five antibiotics belonged to four class, and they included to standard triple therapy. The susceptibility patterns are summarized in table, 3. Out of 8 GAB isolates susceptibility were evaluated, one isolate (isolate 3) exhibited multiple resistant pattern giving the overall Multidrug resistant (MDR) rate of 12.5%. While, the rest of the isolates were susceptible in various degrees (27%, 12.5%, and 25%) to AMO, CLA, and LEV respectively. On the other hand, all GAB isolates were recorded absolute resistance rate (100%) to both of MTZ and TET.

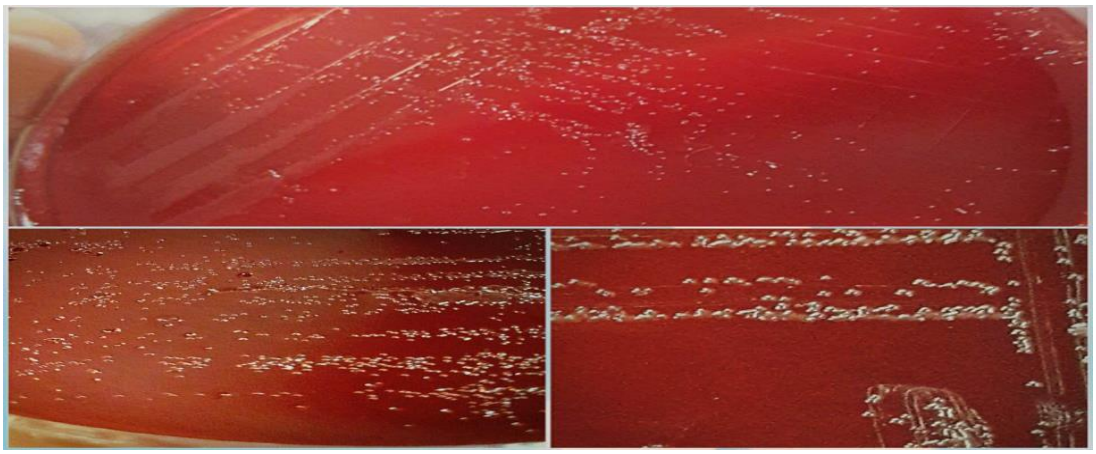

Figure 1. The gastritis associated bacteria isolates colonies on supplemented Colombia agar

Table 3. Antibiotics susceptibility test for gastric associated bacterial isolates

Antibiotics	Break point	Inhibition (mm)	Resistance (%)
Amoxicillin (25 µg)	**≥21 < 16**	12-28	3 (37.5%)
Metronidazole (5 µg)	**≥ND < 21**	9-17	8 (100%)
Tetracycline (30 µg)	**< 17**	8- 15	8 (100%)
Clarithromycin (15µg)	**≥ND < 21**	19-33	1 (12.5%)
Levofloxacin (5 µg)	**≥19 < 17**	15-29	. 2 (25%)

PCR identification of Bacterial isolates

Eight GAB isolates that gave the same characteristics of H. pylori under the microscope, colony morphology, and biochemical testing results were included to molecular detection through PCR amplification of H. pylori 16S rRNA (109bp) and ureA (411bp) genes. After multiple opportunities of running PCR, trying different techniques, changing protocols, and DNA extraction methods, no genes were detected, and no isolate gave positive results (Fig. 2). The isolates 3 and 9 which showed significant morphological similarities to H. pylori and were recovered from the biopsies of severe illness cases, were subjected to identified by amplification of the bacterial universal 16S rRNA gene (Fig.3).

The alignment for the sequence of isolate 3 was revealed high matching with the universal strain (CD-253) sequence which is recorded on NCBI as Achromobactyer xylosoxidans species (accession number: JQ724537.1) at 97% query cover of 98% identify and 0% gaps.

As for the variations seen for the alignment of sequence isolate 9, was revealed high matching with the universal strain (Pb41) sequence which is recorded on NCBI as Klebseilla oxytoca species (accession number: KU761531.1) at 100% query cover of 97% identify and 1% gaps.

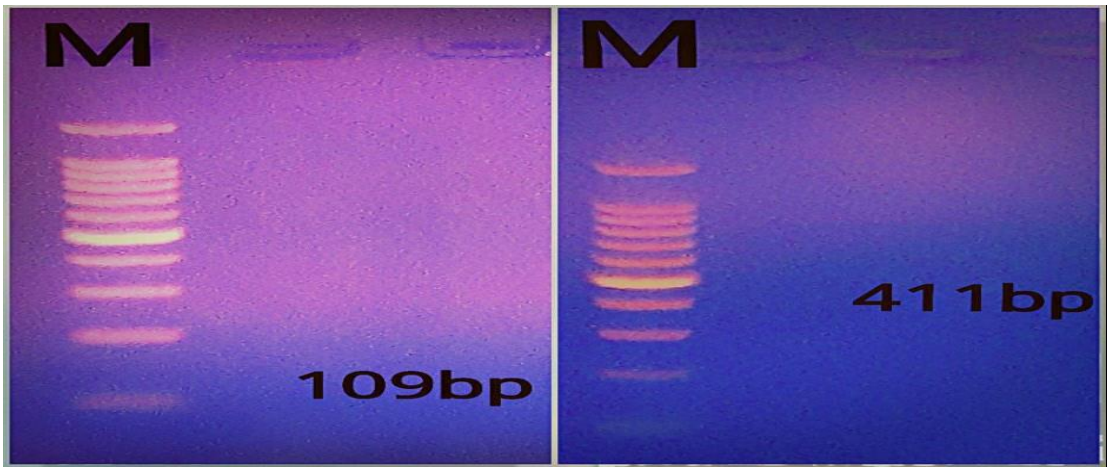

Figure 2. Electrophoresis of PCR product for amplified 16S rRNA and ureA genes of the "thought to be H. pylori" isolates, M-100bp Ladder, A. negative results no 16S rRNA gene detected B. also negative results no ureA gene detected. Electrophoresis was performed on 1% agarose, 70volt/cm for 90min.

Figure 3: Electrophoresis of PCR product ~ 1500 bp of the universal 16S rRNA gene, M-100bp Ladder, Lane (1) isolate 3, Lane (2) isolate 9. Electrophoresis was performed on 1% agarose, 70 volt/cm for 90min.

Discussion

Helicobacter pylori infection represents a key factor in the etiology of various gastrointestinal diseases ranging from asymptomatic gastritis to stomach carcinoma (2). For best management of H. pylori related diseases, the accurate diagnosis is required particularly for infection treatment course. There are various diagnosis methods used for diagnosis of H. pylori in different subjects, precisive detection of this bacterium in different obtained clinical specimens corresponding to the successful therapeutic practice's strategies. Traditionally noninvasive and invasive diagnosis techniques are followed, and each one has certain advantage and disadvantages (5). The invasive diagnosis are endoscopic biopsy dependent methods, and noninvasive are followed to avoid endoscopy (4). Nevertheless, cultural invasive diagnosis based on using highly selective media with several supplements to support bacterium growth and thrive, due to the fastidious and slow growth rate of this bacterium, and its special incubation conditions, such as 10% CO2 and high humidity (6). Also, great care is required during collection and transportation of suspected biopsy specimens to insure successful isolation. Several selective agar media have been used for the isolation of H. pylori from fresh biopsies. Columbia agar medium is one of the most frequently recommended base media to propagate H. pylori culture in routine diagnosis (22,23). Columbia agar supplemented with 7% fresh horse blood used as a base medium with the addition of Dent's supplement, which used to inhibit contaminated flora without loss of H. pylori recovery (20). Multiple biopsies samples rather than a single antral biopsy needed in order to increase the sensitivity and specificity of culture in the diagnosis of the H. pylori (24). Antibiotic resistance is the main reason for failure to eradicate H. pylori infection, the antibiotic resistance of H. pylori has significantly changed over time (25). The obtained GAB isolates were included to the

susceptibility testing based on traditional first line triple therapy regimens used for eradication of H. pylori (26). The obtained pattern of resistance to MET and TET was unexpected even though the resistance to antibiotic drugs may change over time in addition to the resistance depending on the geographical area (27), but the wide shift toward absolute resistance is abnormal, should not be considered for therapy due to the recorded high resistance rate (100%) to both antibiotic. The globally recorded resistance rate to MET does not exceed 80% (28). A study reported discrepancy between in vitro MET resistance and treatment outcome, this may be explained by the changes that occur in the oxygen pressure in the gastric environment, as MET resistant H. pylori isolates become MET-susceptible under low oxygen conditions in vitro (29). As well as, the absolute resistant rate recorded to TET was not in accordance with the publications as the resistance rate of H. pylori to TET is very low (07- 1%) or even absent in most cases (30). The CLA and AMO has resistance amongst the thought to be H. pylori isolates can be considered relatively low. Studied in large scale over the last years have been recommended CLA the drug of choice, although the resistance to CLA seems to be increasing in many geographical areas and the age of the patient affected the resistance rate to CLA (31). The resistance rates to LEV is within normal range 25%, The rates of resistance to metronidazole and clarithromycin are increasing worldwide (32). Based on many publications, standard triple therapies may not be recommended anymore for treatment, due to the high level of resistance to the two key antibiotics of standard triple therapies, CLA and MET, and the different patterns of resistance in different populations (33, 34, 35). Therefore, not considering the resistant isolates to these antibiotics would be incorrect. The discrepancy between the result of this study and the other published studies is due to the consequence of non-isolating H. pylori, rather isolating other bacterial species from the biopsy specimens which was confirmed by molecular diagnosis tools as explained later.

To entitle a sample as H. pylori positive current evidences are indicating the requirement of at least one other positive test in addition to the culture positive result (23) The approach of using PCR genetic invasive identification technique has been proposed, as reliable and predictive confirming diagnosis. However, having positive result from a specific PCR approach can easily replace those time-consuming and expensive tests (36). H. pylori is a microorganism with marked genetic diversity rely using H. pylori housekeeping genes for accurate identification and to evaluate the causes of gastroduodenal diseases (37). Un expected genotyping results was obtained from PCR analysis, not being able to detect the genes (16S rRNA and ureA) of H. pylori, means the ulcer biopsies isolated bacteria may be not H. pylori, or may be the obtained biopsy specimens were not taken from the exact stomach ulcerative area, due to the sensitivity of diagnosis techniques depends on the observer's experience and extent of biopsy sampling (38). Another important issue should be taken in concern, the target gene primer sets not always designed properly, and mis-diagnosis may attribute in some extent to variability in different DNA extraction protocols (39), In this context, a universal approach needs to be recommended to produce reliable results, at least in clinical settings. As one of global housekeeping genes, 16S rRNA was used, (40). However, recently the sequencing of the 16S rRNA gene present in all bacterial species has been a useful tool for identification, it is time consuming and gives accurate results having the16S rRNA gene a molecular marker that is general for all the members of this domain. It's more reliable due to the misidentification of microorganisms when using cultural techniques (41). The 16S r RNA sequencing, GenBank BLAST analysis was showed that the isolates were non-H. pylori isolates, rather, one was identified as Achromobacter xylosoxidans and the other Klebsiella oxytoca, with 98% and 97% identical gene sequences respectively. These two bacteria were isolated from gastric walls of infected patients at regions with ulcers, the patients recruited for the study, were complaining of how they have cured their ulcer infection and then got severe re-infected again after a period of time in addition to the massive amounts of medication they were consuming. May be H. pylori died throughout the first line medication which was a combination of antibiotics and PPI, usually taken for 14 days to eradicate H. pylori. The antibiotics cannot function in the acidic environment of the stomach, therefore, PPI is included within therapy regimen to reduce the hydrochloric acid secretion and the gastric juice acidity (42). This reduces antibiotics washout, thereby increasing luminal antibiotic concentration which causes the lining mucosal layers of the GIT fully damaged. After the course of medication, a completely damaged mucosal membrane was left with no viable H. pylori bacteria in addition to the alkaline stomach conditions there remained months after PPI was stopped (43), and this improves the GIT microbiome and environmental pathogens chance to infect the damaged mucosa. Recently many studies have established links between non-H. pylori microbiota and peptic ulceration, and this is part of studies focuses on the relationship between GIT microbiome in some systemic diseases (44). The ones infected with H. pylori are known to be have a higher content of bacteria that belong to the phylum Proteobacteria in their stomach (17), A. xylosoxidans, K. oxytoca, and H. pylori belong to the Proteobacteria. Studies show that people with dysbiosis have inflamed regions throughout the GIT due to antibiotic therapy, have increased epithelial oxygenation which disturbing the anaerobes and leads to an increase in the facultative anaerobiosis (45). Most of the studies that showing A. xylosoxidans isolated from clinical specimens are from patients with cystic fibrosis (46). One possible way for this bacterium can gain stomach colonization, could be from the sputum swallowed by a patient with an A. xylosoxidans lung infection and cause infection in the stomach (47). Achromobacter is highly motile bacterium

that prefers humid environments and can survive in extreme environments such as, the heavy doses of antibiotics (48), and infects those persons with non-protecting lining epithelia and weak gastric mucosa, allowing it to cause infection and eventually gastritis. 15-year time period studies were supported the role of non-H. pylori microorganisms in the development of chronic gastric ulcer to malignant tumors, and highlighted the effects of H. pylori eradication therapy on gastric neoplasia (49). A computerized search algorithm was construct to identify the presence of bacterial DNA within interrogated known cancer genomes, they identified that the type of cancer that harbored the second highest number of bacterial DNA sequences was gastric adenocarcinoma, and found the most common type of bacterial DNA was not H. pylori but instead was Pseudomonas, and due to their high similarity A. xylosoxidans is always misdiagnosed with Pseudomonas (50). The fact that one of the isolates turned out to be A. xylosoxidans not H. pylori, the break points for resistance was not available by the CSLI, instead it was considered one of the organisms that should not be monitored "Unusual organisms which are rarely involved in serious infections" (WHO, 2011). Studies used the breakpoints recommended by CLSI for non- Enterobacteriaceae (51). A study was performed to demonstrated the MDR profile of human derived strains of A. xylosoxidans, intrinsically it was resistance to list of study included therapies, (benzylpenicillin, cefoxitin, cefamandole, cefuroxime, glycopeptides, fusidic acid, macrolides, lincosamides, streptogramins, rifampicin, daptomycin, and linezolid) (52). This proves the extremophilic behavior of bacterium that stays alive while all the other bacteria in the stomach or intestine, which will give them an opportunity to cause infection. Our isolate showed clear resistance to all the antibiotics tested with no inhibition zone.

The seconded isolated bacterium (isolate 9) turn to K. oxytoca, numerous studies have shown that H. pylori eradication second line therapy may cause antibiotic associated hemorrhagic colitis (AAHC), where patients begin with a peptic ulcer and after the repeat courses of medication end up with more severe AAHC. Clinical studies were approved predominance of Klebsiella. oxytoca in biopsy specimens taken from such patient from inflamed colon tissue (53). Recently K. oxytoca has been isolated at a significantly high rate from endoscopic biopsy was taken from severe inflamed gastric mucosal necrotic areas for elderly man, was diagnosed H. pylori positive. Contrast-enhanced computerized tomography scan of abdomen demonstrated diffuse gastric wall thickening and an intramural abscess in the gastric antral wall (54). Genome sequencing of K. oxytoca clinical isolate was revealed a combination of virulence, antibiotic resistance and metabolic genes that assist the species survive and transmit within the human clinical isolate in host and overcome challenges in the harsh habitat such as antibiotics (55). This was realized throughout this study during the antibiotic susceptibility test where the isolates that ended up to be K. oxytoca showed variable results, a few times showing light growth surrounding the CLA and AMO discs, and other times sensitive with inhibition zones which may consider them resistant to these antibiotics resulting in their resistance to the full triple therapy and other times showing a clear inhibition zone. Both of these unusual bacterial isolates had a mucoid appearance, in which this characteristic helps in protection from the tough conditions of the stomach and large intestine enabling its capacity to produce biofilm and surviving throughout the GIT. The ability of a bacterium to produce biofilm is dangerous owing to its difficulty in eradication and having the mucus lining of the GIT suitable for such act (56).

Conclusions

A combination of at least two diagnostic tests with high sensitivity and specificity for diagnosis of peptic ulcer etiological agents.

Ulcerative gastritis is not always related to infection with H. pylori, may be other bacteria such as GIT microbiota or external opportunistic bacteria are the causative agents.

Abuses of H. pylori eradication therapy may consequently change stomach microbiome and lead to more severe ulceration cases

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