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Detection of *H.pylori* cagA gene in patient's with gastroduodenal disease

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Abstract: *Helicobacter pylori* infection is common in the developing countries. The *cagA* gene is a marker of pathogenicity island (PAI) in *H. pylori*. The aim of this study was to determine the prevalence of *cagA* among gastroduodenal disease (peptic ulcer and non-ulcer dyspepsia) in patients of Ramadi-city. A total of 81 gastric biopsy samples, gastric ulcer 10 (12.30%) , duodenal ulcer 18 (22.20%) and non-ulcer dyspepsia 53 (65.40%) by endoscopic examination . *cagA* gene of *H. pylori* was assessed using polymerase chain reaction (PCR). The *cagA* gene was detected by PCR technique in 3 from 10 biopsy specimens culture positive, urease test was showed 7(8.64%) patients, 2(2.5%) patients and 48(59.26%)patients after (2 hr, 6hr and 20 hr) respectively, ELISA positive result was showed 12(14.8%) patients. Urease test is easy test , inexpensive and can be use in endoscopy unit, but non-specific, culture is gold stander for detection of *H.pylori*, ELISA technique use for detection of anti-*H.pylori* IgG , ELISA technique widly used for epidemiology studies. PCR assay used for detection of cagA gene. Using PCR to detect *cagA* gene from culture colony. The potential advantages of PCR include high specificity, quick results and the ability to type bacteria without the requirement for special transport conditions..

Key Words: Cag A gene. Pepti ulcer. Gastric biopsy. Invasive methods.

Introduction:

Helicobacter pylori , previously named *Campylobacter pyloridis*, is a Gram-negative,microaerophilic bacterium found in the stomach. It was identified in 1982 by Barry Marshall and Robin Warren, who found that it was present in patients with chronic gastritis and gastric ulcers, conditions that were not previously believed to have a microbial cause. It is also linked to the development of duodenal ulcers and stomach cancer. However, over 80 percent of individuals infected with the bacterium are asymptomatic and it has been postulated that it may play an important role in the natural stomach ecology.[1]

More than 50% of the world's population harbor *H.pylori* in their upper gastrointestinal tract. Infection is more prevalent in developing countries, and incidence is decreasing in Western countries. *H. pylori*'s helix shape (from which the generic name is derived) is thought to have evolved to penetrate the mucoid lining of the stomach.[2][3]

There are two classical secreted virulence factors present in *H. pylori* the vacuolating cytotoxin (VacA) and the CagA protein encoded by the cytotoxin-associated gene-pathogenicity island (cagPAI). VacA interacts with numerous host surface receptor molecules and can trigger various responses, including pore insertion into the cell membrane, modification of endolysosomal functions, cell vacuolation, apoptosis and immune inhibition) (4). The cagA gene (cytotoxin-associated gene) is considered a marker for the presence of one pathogenicity island of about 40 kbp. The structure of the gene reveals a 59 highly conserved region and a variable 39 region, in which the presence of a variable number of repeat sequences results in a protein with a molecular mass of 120 to 140 kDa (5).

CagA-producing *H. pylori* strains induce inflammatory cytokines, especially interleukin-8 (6) . The presence of cagA is associated with duodenal ulcer, gastric mucosal atrophy, and gastric cancer and the majority of *H. pylori* strains can be classified into one of two groups (cag positive or cag negative), based on the possession of the cagA gene and the associated genes in the cag pathogenicity island (7).Other well-known pathogenicity-associated processes include flagella-driven *H. pylori* motility, urease-triggered neutralization of pH, several adhesins. There are also numerous other reported marker genes for *H. pylori*-induced disease development (e.g. iceA and dupA), although their biological function is widely unclear.

Patients and Methods :

Between October 2011 to May 2012, a total of 81 adult patients examined in the gastro endoscopy unit (53 male and 28 female) with in the mean age (35.89) year range between (14-78 years) .

These patients with dyspeptic symptoms were referred by their physician for diagnostic upper gastrointestinal endoscopy in Ramadi Teaching Hospital. The patients had not received non-steroidal anti-inflammatory drugs(NSAID), proton pump inhibitor or antibiotics before to examination. Patients' history and endoscopy findings were obtained from case report.

Gastric biopsies were taken from patients suffering from duodenal ulcer (DU), gastric ulcer (GU) and non-ulcer dyspepsia (NUD).

Detection and diagnosis of *H.pylori* : Testing for *H. pylori* infection has become a very important part of the diagnostic process for gastric and duodenal inflammatory disease, since the presence or absence of infection determines the type of treatment to be applied. Testing is also a useful means of monitoring the effectiveness of courses of antimicrobial treatment. A number of different diagnostic test methods, both invasive and non-invasive, are available.

Non-invasive methods: Several techniques for non-invasive diagnostic tests for *H. pylori* have been developed and tests based on one of these are in widespread use.

-**Serological tests** : People infected with *H. pylori* generally have specific IgG antibody circulating in their blood and these can be detected by serological tests. This test is required of 3-5 ml blood aspiration from each patient's. A number of commercially available tests have been developed, mostly based on IgG detection, which is considered slightly more sensitive and specific. Antibody detection is usually by using ELISA technique. All study specimens were submitted to ELISA technology for detection of *H. pylori* IgG antibody by using (DRG International Inc., U.S.A.).

Invasive methods: The collection of biopsy specimens from antrum regions of the stomach and duodenum during invasive endoscopy (Pentax EG-2470 K , Japan) is considered to be the reference method for diagnosing *H. pylori* infection. The biopsy material can be examined using one, or more, of three different test methods.

-**Urease test** : A colorimetric test for the presence of urease enzyme activity (indicative of *H.pylori* infection) in the biopsy sample. This method can be used to give a rapid indication of infection at the time of the biopsy. One piece of antral biopsy was inoculated in urease agar tube. The presence of urease was indicated by color change from yellow to pink (8).

-**Culture** : For culture, each antrnal biopsy specimen was to be placed in brain heart infusion broth as transport media , for each specimen before cultivation , sections were done for each biopsy with sterile needle to facilitate release of *H. pylori.* Biopsy specimen was carefully cultivated on to brucella chocolate agar containing 7.5% horse blood and campylobacter supplement. The plates were incubated at 37 °C under microaerophilic and high humidity conditions provided by a gas pak generation kit for 4-7 days (9).

Molecular part of this study:

Extraction of Genomic DNA from study bacteria:

DNA extraction was performed using the Wizard® SV kit (Promega, Madison, USA). PCR amplification reaction was used according to AL-Jobori, (19).

***cagA* detection by PCR :** All the samples of bacterial culture were employed for DNA extraction which were assayed by PCR amplification process using priemer prepared by Bioneer Korea. The primers 5-AGTAAGGAGAAACAATGA-3 and 3-AATAAGCCTTAGAGTCTTTTTGGAAATC-5 . These primers were designed to amplify the variable region of the *cagA* gene. Depending on the type and number of repeats present in the sequence of the gene. These forward and reverse primers, CagA/ConF (5-AGTAAGGAGAAACAATGA-3) and CagA/Con-R(3-AATAAGCCTTAGAGTCTTTTTGGAAATC-5), amplify a 1320-bp . PCRs were performed in a

volume of 20 µl solution containing (Master Mix) (1U Taq DNA polymerase, 250µM (each) dATP, dGTP, dCTP, dTTP, 10mM Tris-HCl (9.0),30mM KCl, 1.5 $MgCl_2$, stabilizer and tracking dye. Add 5ng template DNA , 5pmole from primer and add distille water to AccuPower PCR tube to a total volume 20µl. PCR was performed by a (Maxy Gene, USA) thermocycler , under the following conditions : involving initial denaturation 5 minutes at 95 ^oC, followed by 42 cycle each of denaturation 1 minute at 95 ^oC, annealing 1 minute at 65 ^oC, extension 1 minute at 72 ^oC and a final extension step at 72 ^oC for 1 minute. PCR amplification was performed in duplicate for each DNA sample. The products of amplification were examined by electrophoresis on 2% agarose gels according to standard procedures (36).

RESULTS :

In the presents study a total of 81 patients ,Male 54/81(67%) , Female 27/81(33%) . All patients were proved to have gastric ulcer 10 (12.30%) , duodenal ulcer 18 (22.20%) and non-ulcer dyspepsia 53 (65.40%) by endoscopic examination (Figure 1). The mean age patients was (35.9) years with a range of (14-78) years and the standard deviation is (±14.95).

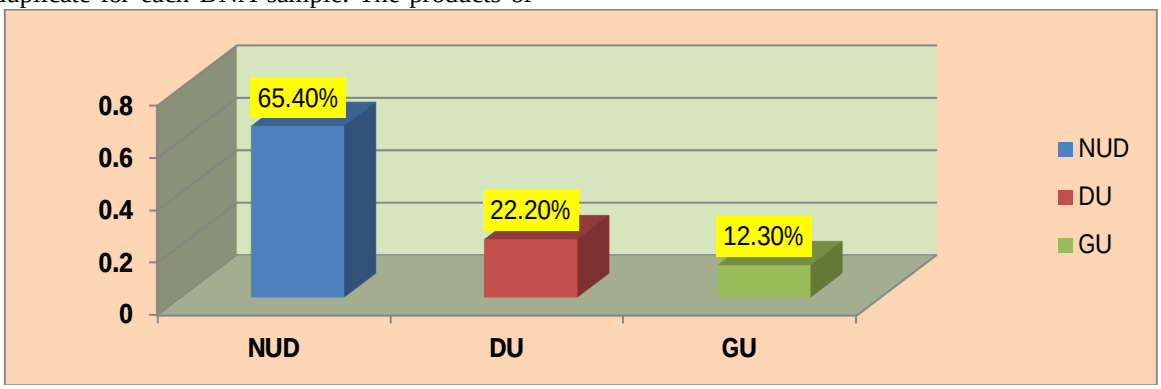

Figure (1):Distribution of clinical specimens according to endoscopic examination.

Urease Test: In this figures (2a-2b) show the positive and negative result .

Fig.(2a): positive urease

Fig.(2b):Negative urease

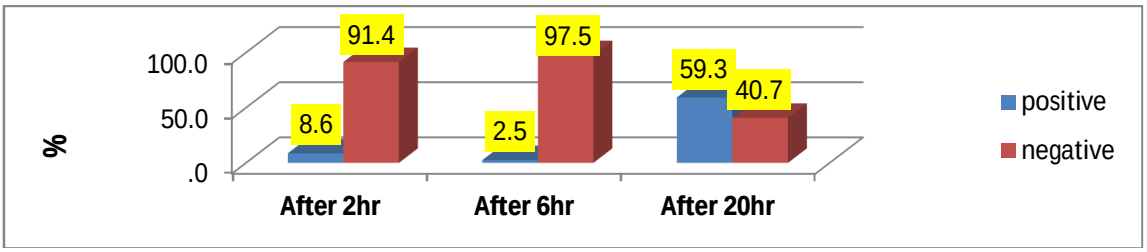

Figure (3): The result of urease positive and negative test in patients according to the time.

The positive results appear after (2 hr, 6hr and 20 hr) were7 patients(8.6%) , 2 patients (2.5%) and 48 patients (59.3%)respectively (Figuer 3).

Culture: Out of 10 (12.34%) specimens showed growth of *H.pylori* on Brucella chocolate agar. 5 (6.17%) of them were from patients with duodenal ulcer (DU) ,3 (3.7%) were from patients with gastric ulcer (GU) and 2 (2.5%) were from patient's with non-ulcer dyspepsia (NUD) (table 1).

Table (1): The result of *H. pylori* positive and negative culture regarding patients status.

Type of specimen	No. of specimen	Culture positive cases No. (%)	Culture negative cases No. (%)
Gastric ulcer(GU)	**10**	**3 (3.7%)**	**7 (8.64%)**
Duodenal ulcer(DU)	**18**	**5 (6.17%)**	**13 (16.05%)**
Non-ulcer dyspepsia(NUD)	**53**	**2 (2.5%)**	**51 (63 %)**
Total no.	**81**	**10**	**71**

Although careful in the whole steps of culturing including careful media preparation , transport , incubation atmosphere and identification steps**.** The colony shaped of *H. pylori* on Brucella chocolate agar after 4-7 days of incubation appeared as water droplets, small, convex and translucent.

ELISA results: These tests were used for the detection of *H. pylori* IgG titer in patients with *H. pylori* infection. 12 (15%) out of 81 cases showed positive by ELISA technique in serum samples (figure 4).

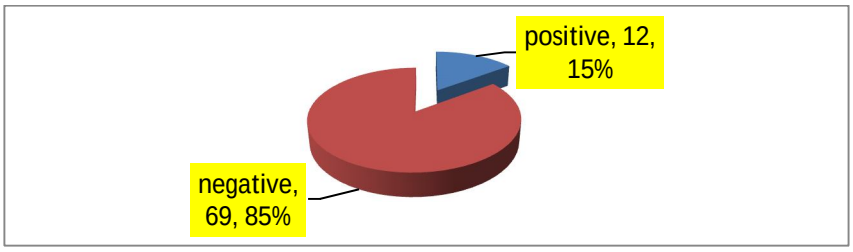

Figure (4): Distribution of ELISA results.

The positive cases were showed by ELISA technique had, significantly urease positive test. The statistical analysis showed that the *H. pylori* positive patients showed significantly higher titers of anti-*H. pylori* IgG (1.78 ± 0.38) in serum samples than *H. pylori* negative subjects (0.51 ± 0.12) ($p<0.01$).

Results of PCR: Ten (12,34) out of 81 *H. pylori* isolates by culture method from gastric biopsy samples. Only 3(30%) isolates gave positive results for cagA gene by PCR technique (Table 2).

The statistical analyses showed significant difference between culture and PCR considering P value ($p< 0.01$). Three out of *H. pylori* DNA extracted showed cagA positive from bacterial colonies revealed DNA diagnostic band with 1320 bp (figure 5).

Table (2) PCR result for *H pylori* genome extracted from bacterial culture.

PCR positive for bacteria	PCR negative for bacteria
3 (30 %)	**7 (70 %)**

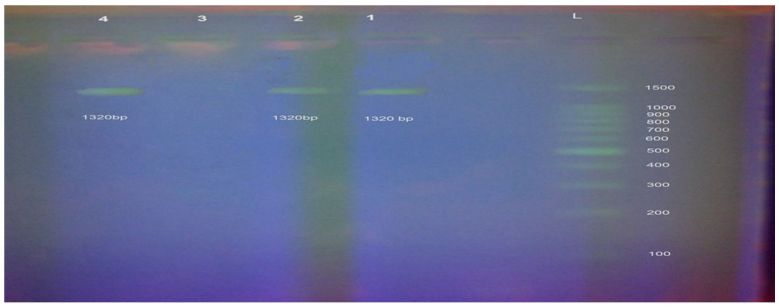

Fig. (5) PCR products of cagA gene positive band extracted from bacterial colonies (1, 2 and 4) by using agarose 1.5% , Red safe Nucleic acid staining solution and ladder PCR.

Discussion:

Helicobacter. pylori is a Gram-negative bacterial pathogen that selectively colonizes the gastric epithelium of approximately half of the world's population (10) . The most common outcome of *H. pylori* infection is chronic asymptomatic gastritis (11); however, long-term colonization with *H. pylori* significantly increases the risk of developing gastro-duodenal diseases. Among infected individuals, approximately 10% develop peptic ulcer disease, 1-3% develop gastric adenocarcinoma, and less than 0.1% develop mucosa associated lymphoid tissue (MALT) lymphoma (12) . Accordingly, *H. pylori* is classified as a Type I carcinogen, and is considered to be the most common etiologic agent of infection-related cancers, which represent 5.5% of the global cancer burden (13).

H. pylori virulence factors, their interaction with the host and consequences in pathogenesis. These include the role of bacterial genetic diversity in host colonization and persistence, outer membrane proteins and modulation of adhesin expression, new aspects of VacA functions and CagA, its phosphorylation dependent and -independent cellular effects. This article will also review the recent novel findings on the interactions of *H. pylori* with diverse host epithelial signaling pathways and events involved in the initiation of carcinogenesis, including genetic instability and dysregulation of DNA repair.

The cagA gene which is encoded for by cagPAI has been described as a marker for pathogenic *H. pylori* strains and has genes homologous to those of the type IV secretory pathway. This pathway encodes a secretion system involved in the export of virulence determinants and influences the secretion of IL-8, involved in the severity of the disease. Furthermore, studies suggested that there is a correlation between the prevalence of cagA positivity and peptic ulcer disease (16).

The cagA gene product CagA is delivered from the bacterium into the cytoplasm of the bacterium-attached gastric epithelial cell via the type-IV secretion system. Upon membrane localization and subsequent tyrosine phosphorylation by Src family kinases, translocated CagA functions as a scaffolding adaptor that interacts with a number of host proteins involved in cell signaling in both tyrosine phosphorylation-dependent and -independent manners. Of special interest is the interaction of CagA with the SHP-2 tyrosine phosphatase, of which gain-of-function mutations have recently been found in human malignancies. Through the complex formation, SHP-2 is catalytically activated and induces morphological transformation that is associated with increased cell motility. In addition to the perturbation of intracellular signaling, CagA disrupts the apical junctional complex that regulates the cell–cell contact and maintains the integrity of the epithelial structure. (40).

The urease test remain inexpensive, fast and widely available. It is well known that urease test is highly specific for *H. pylori* infection and is commonly used for the detection of *H. pylori* infection at endoscopy (17) .

Regarding urease test result, this study was in agreement with (18,19) and disagreement with those observed by (20), decreased urease activity, caused either by recent ingestion of antibiotic agents, bismuth compounds, proton pump inhibitors, bile reflux or biopsy specimen were taken from non- targeted area (17)

Urease test is not sensitive and may non specific, because of in some cases showed false positive results of urease test may be caused

by cross reaction with other urease-positive bacteria like, *klebsiella* spp. and *Proteus* spp. (9,21).

Culture is consider to be the most specific method for identification of *H. pylori* infection (100 % specific), its sensitivity has been found to be low even under most favorable conditions, but remains the gold standard technique. (22). The detection of *H. pylori* is critical due to the high prevalence of this species, its worldwide distribution, and the large number of individuals carrying this species (23).

Ten (12.34%) out of total cases appeare positive culture, while other were negative culture, this result was agreement with (19), while disagreement with those observed by (24). This low isolation rate may be due to its a fastidious slow growing bacterium, it requires 4 to 7 days to grow in rich media and it requires specific culture conditions (9), irregular distribution in gastric mucosa, fastidious nature, mucosal weaken, (25),intestinal metaplasia, administration of antibiotics (due to some other infection or protozoal infestation) and proton pump inhibitors (26).

Another factors that may influence the success rate of culture involves the transport conditions from the endoscopy room to the laboratory and the patient's oropharyngeal flora can also markedly reduce the isolation rates and the colony numbers of *H. pylori* (27).

ELISA is one of the most extensively employed tests, because it is relatively inexpensive, quick, easy to perform, suitable for screening large populations, and capable of detecting class-specific immunoglobulins (28). ELISA has been widely used in epidemiological studies, including retrospective studies to determine the prevalence or incidence of infection (28).

Twelve (14.81%) out of total cases appeare positive serological method, while other were negative, this result was agreement with (14), while disagreement with those observed by (43). This low result due to antibiotics are frequently approved for common infections (upper respiratory infections, urinary tract infections, etc.). Monotherapy with commonly used antibiotics, such as metronidazole or clarithromycin, can achieve *H. pylori* eradication rates in up to 17–20% of patients (44). Serum antibody tests remain positive for a significant period, or perhaps indefinitely, after *H. pylori* eradication (45). Remote ingestion of certain antibiotics in potential study candidates could therefore result in eradication of *H. pylori* **(** 45).

Additional, ELISA technique may not able to tell between present (active) from past infection and cannot be used to indicate the clearance of *H. pylori* from the stomach because of the antibodies were may stay at the same level or decline very slowly even after suppression of the bacteria that lead to false positive result (9,29).

Lower specificity of serological tests observed in certain studies may have largely been explained by the inclusion of patients previously treated for *H. pylori* infection or having received antibiotics for the treatment of various intercurrent infections (30). Furthermore, differences in test accuracy can be explained by the use of various antigen preparations or by differences in infecting strains that result in different immune responses. This has led to the general recommendation that any serological test for *H. pylori* should be validated and standardized locally before use. In many instances, this will imply the adjustment of the cut-off values recommended by the manufacture (31).

In the present study, two cases were negative by rapid urease test, but had significant IgG levels. This may be due to past infection or patchy distribution of organism in the stomach which was responsible for negative rapid urease test (32).

A Polymerase chain reaction (PCR) has been successfully used to detect *H. pylori cagA* virulence genes in bacterial colonies from gastric biopsies. The potential advantages of PCR include high specificity and sensitivity, quick results and the ability to type bacteria without the requirement for special transport conditions (15) . The assay used for follow suppression therapy , PCR requires specialized laboratory facilities and is not generally available as a routine diagnostic test (33), and for the detection of *Helicobacter pylori* in bacterial growth from gastric biopsy specimens with specific primers for cagA gene amplification was compared with other routine invasive methods (culture, rapid-urease test and histological analysis) with samples from a group of 81 consecutive dyspeptic patients. Bacteria were found in 10 (12,34%) of the patients by culture, but 3 samples from 10 samples were showed positive results by PCR. Limitations of PCR methods include the propensity for false - positive results in part due to the detection of cDNA from non-*H. pylori* organisms. False-

negative results may also occur due to a low number of organisms or to the presence of inhibitors (41). that cause vanish DNA bands or PCR inhibitors generally exert their effects through direct interaction with DNA or interference with thermostable DNA polymerases. Direct binding of agents to single stranded or double-stranded DNA can prevent amplification and facilitate co-purification of inhibitor and DNA. Inhibitors can also interact directly with a DNA polymerase to block enzyme activity. DNA polymerases have cofactor requirements that can be the target of inhibition. Magnesium is a critical cofactor, and agents that reduce Mg2+ availability or interfere with binding of Mg2+ to the DNA polymerase can inhibit PCR. The presence of inhibitors in samples has been the focus of much of the published literature. Common sample types known to contain inhibitors include blood, fabrics, tissues and soil . Other important sources of inhibitors are the materials and reagents that come into contact with samples during processing or DNA purification. These include excess KCl, NaCl and other salts, ionic detergents such as sodium deoxycholate, sarkosyl and SDS, ethanol and isopropanol , phenol and others (37).

This study in agreement with (38), while disagreement with(39). The cagA gene primer did not detect in all of the *Helicobacter spp.*,but the cagA is present in 20 -30% from *H. pylori* spp. When the infection was contracted is important, in our opinion, especially because *H. pylori* is known as a life-long infection and it colonizes the human stomach for a long period of time until it causes severe infections, individuals might been infected recently and more severe complications have yet to be produced. The other consideration will be where they acquired the infection (42).

In conclusion,culture and PCR assay for detection of *H. pylori* cagA is more reliable in biopsies than urease test .

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الكشف عن جين (cag A) البكتريا الملتوية البوابية في المرضى المصابين بامراض المعدة والاثني عشري

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الخلاصة:

عدوى البكتريا الحلزونية هي مرض شائع في البلدان النامية. الcag جين هو مؤشر على PAI في البكتريا الحلزونية. الهدف من هذه الدراسة هو تحديد انتشار ال cag ضمن مرضى القناة الهضمية (قرحة القناة الهضمية و عسر الهضم غير القرحي) في مرضى مدينة الرمادي. من مجموع 81 نموذج خزعة معدية, 10 لديهم قرحة معدية, 18 قرحة الاثني عشري و53 عسر هضم باستخدام فحص الناظور. Cag جين البتكريا الحلزونية يحدد باستخدام تفاعل البلمرة المتسلسل (PCR). ويتم الكشف عنه باستخدام PCR في 3 من 10 نماذج خزعية موجبة الزرع, وفي اختبار اليوريز شوهد 7 مرضى, 2 مريض و48 مريض بعد (2 ساعة, 6 ساعة و20 ساعة) بالتعاقب. نتائج الفحص السيرولوجي الموجبة هي 12 مريض.اختبار اليوريز هو فحص سهل , رخيص الثمن وبالامكان اجراءه داخل وحدة الناظور, لكنه فحص غير نوعي. الزرع هو الفحص الذهبي للكشف عن البكتريا الحلزونية. الفحص السيرولوجي يستخدم للكشف عن الاجسام المضادة للبكتريا IgG , الفحص السيرولوجي استخدامه واسع في الدراسات الوبائية. فحص PCR يستخدم للكشف عن cagA جين من المستعمرات الزرعية, وهو ذات فرص ممكنة وقوية ويكمن نوعية عالية, نتائج سريعة وقدرة الكشف عن اي نوع بكتيري بدون الحاجة اوضاع ونواقل خاصة.