Duplex PCR primers for detection of *Helicobacter pylori* DNA directly from gastric biopsy samples

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Abstract

In order to avoid PCR inactivation because of nucleotides variability in primers annealing sites in template DNA molecules and confirmation the presence of medically important bacterium *Helicobacter pylori* in gastric biopsy samples with high accuracy, relatively low cost and easily, several primers pairs analyzed for duplexing capacity. One of 16s ribosomal RNA gene, and one of *glm*M (*Ure*C) gene of this bacterium have duplexing capacity. Those primers are designed separately to detect the presences of *H. pylori* with high efficiency. Dimerization analysis resulted in allowable degree to use in duplex PCR, and the laboratory results confirmed applicability of this hypothesis. The present study concluded that the combination of these two primers is suitable for accurately detection of these bacteria directly from gastric biopsy specimens.
Specialty: Molecular Microbiology, Computational biology

ضم بوادئ تفاعل الكوثرة المزدوج للكشف عن كميات دقيقة من DNA بكتريا الملويات البوابية (*Helicobacter pylori*) مباشرة من عينات الخزع المعدية

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الكلمات المفتاحية: Helicobacter pylori, عينات الخزع, علم الاحياء الحاسوبي, تفاعل الكوثرة المتعدد.

الخلاصة

لاجل تلافي فشل تفاعل الكوثرة بسبب التقلب في النيوكليوتيدات في مواضع ارتباط البوادئ بجزيئات DNA القالب و تاكيد وجود بكتريا الملويات البوابية (*Helicobacter pylori*) المهمة طبيا في عينات الخزع المعدية بدقة عالية و كلفة مقبولة نسبيا و بطريقة سهلة , تم تحليل عدد من البوادئ لايجاد امكانيتها في احداث تفاعل الكوثرة المزدوج وقد وجد ان احد البوادئ المتخصصة بتضخيم جين 16s ribosomal RNA لهذه البكتريا و كذلك احد البوادئ الخاصة بتضخيم جين *glm*M (*Ure*C) لنفس البكتريا لهما القابلية في احداث تفاعل الكوثرة المزدوج Duplex PCR سوية في ان واحد. و ان هذه البوادئ قد تم تصميمها مسبقا لغرض الكشف عن هذه البكتريا بكفائة عالية كل على حدة و قد بينت النتائج ان فحص الازدواج الذاتي و المتباين قد افضى الى درجات مسموح بها لاجراء تفاعل الكوثرة المتعدد و ان النتائج التجريبية اثبتت هذه الفرضية. و يستنتج من الدراسة الحالية ان الجمع بين هذين البادئين ملائم للكشف الدقيق عن و جود هذه البكتريا في عينات الخزع المعدية.

التخصص: علم الاحياء المجهرية الجزيئي, علم الاحياء الحاسوبي.

Introduction

Helicobacter pylori are a Gram negative bacterium that is highly adapted for persistent colonization of the human stomach. Although most *H. pylori* infected people remain asymptomatic. The presence of these organisms is considered a risk factor for gastric adenocarcinoma, peptic ulcer disease and gastric lymphoma (1). Molecular methods are widely used for the diagnosis of *H. pylori* infection as well as analyses of diversity, virulence, persistence and resistance patterns of these bacteria (2).

PCR-based methods have been developed to detect the organism directly in clinical specimens. The targets of these PCR methods include the 16S rRNA gene, random chromosome sequence, 26-kDa species-specific antigen (SSA) genes, urease (*ure*A) gene, and *glm*M (*ure*C) gene. *UreC* gene encodes for a phosphoglucosamine mutase; this gene is unrelated to urease production, so it was renamed *glm*M. This gene is considered a "housekeeping" gene, and it participates directly in cell wall synthesis (3).

The worldwide distribution and high level of prevalence and the importance of associated pathologies makes the elimination of *H. pylori* a very useful approach in order to treat and control these gastroduodenal diseases, since its eradication results in a marked reduction in the rate of recurrence of duodenal and gastric ulcer. Methods that accurately detect *H. pylori* infection in dyspeptic patients are therefore of major importance. Direct demonstration of *H. pylori* in gastric biopsy specimens is possible through the use of culture, histological examination with several stains, and assays for rapid urease activity. Culture, although labor-intensive because of the fastidious nature of the organism, is a very sensitive detection method with the advantages of specifically detecting *H. pylori* and making strains available for susceptibility testing. All these endoscopy-based methods require gastric biopsy specimens and are thus classified as invasive methods (4)**.**

Due to the presence of some sequence variation in bacteria, molecular detection may fail in detection of some strains, so to avoid this possibility, duplex PCR detection used to reduce this phenomenon by confirmation with additional primers. The present study aimed to develop an easy, relatively cheap and confirmed method for the detection of this organism with Duplex PCR.

Material and methods

Source of specimens

Gastric biopsy specimens collected from patients with gastric symptoms attending to Al-Husain general hospital in Karbala in Iraq.

Computer software

Oligo analyzer tool used to assess primers dimerization potential (http//www.idtdna.com/analyzer/applications/oligoanalyzer/) (5).

Primers sequences

Fifteen published sequences for amplification of 16S rRNA gene, and three sequences used to amplify *glm*M gene of *H. pylori* listed in Tables 1 and 2 are analyzed for duplexing potential.

Table1: 16S rRNA gene detection primer sequences of *H. pylori.*

No.	**16S rRNA primer sequence**	**Annealing temperature**	**Product size (bp)**	**Reference**
1*	TGGCAATCAGCGTCAGGTAATG GCTAAGAGATCAGCCTATGTCC	55°C	522	6,7
2	GCGCAATCAGCGTCAGGTAATG GCTAAAGAGATCAGCCTATGTCC	60℃	118	8
3	CTGGAGAGACTAAGCCCTCC ATTACTGACGCTGATTGTGC	60 C	109	9
4	TAAGAGATCAGCCTATATGTCC TCCCACGCTTTAAGCGCAAT	56℃	534	10
5	CGTTAGCTGCATTACTGGAGA GAGCGCGTAGGCGGGATAGTC	60°C	295	11
6	GTCATGACGGGTATCC ACTTCACCCCAGTCGCTG	55°C	12,00	12
7	AGAGTTTGATYMTGGC TACGGYTACCTTGTTACGA	50°C	1,500	13
8	GCTATGACGGGTATCC GATTTTACCCCTACACCA	55°C	400	14
9	TGCGAAGTGGAGCCAATCTT GGAACGTATTCACCGCAACA	60°C	118	15
10	TAAGAGATCAGCCTATGTCC TCCCACGCTTTAAGCGCAAT	56°C	534	16
11	CTGGAGAGACTAAGCCCTCC AGGATCAAGGTTTAAGGATT	55°C	446	17
12	CTGGAGAGACTAAGCCCTCC ATTACTGACGCTGATTGTGC	62°C	110	17
13	GCTAAGAGATCAGCCTATGTC CCGTGTCTCAGTTCCAGTGT	55°C	118	18
14	GCGACCTGCTGGAACATTAC CGTTAGCTGCATTGGAGA	55°C	139	19
15	GTGTGGGAGAGGTAGGTGGA GTTTAGGGCGTGGACTACCA	56°C	163	20

* Selected primer for duplexing.

Table 2: *glm*M gene detection primers of H. *pylori*.

No.	*glm*M primers	Annealing temperature	Product size (bp)	Reference
1*	AGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	55°C	294	17, 21, 23
2	GGATAAGCTTTTAGGGGTGTTAGGGG GCTTACTTTCTAACACTAACGCGC	56°C	294	24,12
3	GCATTCACAAACTTATCCCCAATC GGATAAGCTTTTAGGGGTGTTAGGGG	58°C	140	3

* Selected primer for duplexing

DNA extraction and PCR amplification conditions

Total DNA extracted directly from gastric biopsy samples using tissue protocol (Geneaid, Korea), the final volume of DNA extraction product was 200 µl, with final concentration 1.25 ng/µl.

PCRpremix™ kit from (Bioneer, Korea) used to amplify mentioned genes.

Total reaction volume of 20 µl containing, 3 µl of extracted DNA, 0.7 µl of 10 pmol/µl of each forward and reverse primers for 16S rRNA gene, 1 µl of 10 pmol/ µl of each forward and reverse primers for *glm*M gene in addition to 14 µl of molecular biology grade water then the mixture added to lyophilized PCRpremix™ formula.

The following conditions used to amplify both of *H. pylori* 16S rRNA and *glm*M genes with 38 cycles, Initial denaturation 94 for 5 minutes , each cycle with denaturation at 92°C for 30 second, primers annealing at 55°C for 40 second, primers extension at 72°C for 40 second, final extension at 72°C for 5 minutes. PCR products were detected using 2% agarose gel electrophoresis.

Results

Duplexing primers selected from several previously mentioned primers listed in Tables 1 and 2. Two sets of primers were selected for *H. pylori* detection according to annealing temperature, PCR product length and dimerization potential. One of these primers is specific to amplify 16S rRNA gene with 522 bp product mentioned with number 1 in Table 1. And the second primer is *glm*M (*Ure*C) gene, giving 294 bp products mentioned with number 1 in Table 2.

Selected primers potential for self and hetero dimerization were checked as mentioned in Table 3 that all dimerization deltas G values for all probable dimerization forms doesn't exceed threshold value (-7 kcal/mol).

Table (4) Summarize the annealing strength possibilities for self and hetero dimerization among selected primers for duplexing *H. pylori* 16S r RNA gene and *glm*M (*ure*C) genes. All delta G values don't likely to be high to inactivate duplex PCR reaction. Figure 1 illustrates Agarose gel electrophoresis results of duplexing primers for 16S rRNA, 522 bp and *glm*M, 294 bp for detection of *H. pylori*.

Table (3) Dimerization potential of selected duplexing primers.

Combinations	**Dimerization probability**	**Comments**
16S rRNA -F with 16S rRNA-R	5'-CAATCAGCGTCAGTAATGTTC-3' ||| 3'-CCTGTATCCGACTAGAGAATC-5' dG: 0.16 kcal/mol	Both primers anneal to each other with impossible extension manner.
16S rRNA -F with *glm*M- F	5'-CAATCAGCGTCAGTAATGTTC-3' : : ||| : 3'-TTTGGGGATTGTGGGGATTTTCGAA-5' dG: -1.53 kcal/mol	Both primers anneal to each other with impossible extension manner.
	5'-CAATCAGCGTCAGTAATGTTC-3' : : : ||| 3'-TTTGGGGATTGTGGGGATTTTCGAA-5' dG: 0.31 kcal/mol	Delta G 0.31 kcal/mol is very small.
	5'-CAATCAGCGTCAGTAATGTTC-3' : ||| : 3'-TTTGGGGATTGTGGGGATTTTCGAA-5' dG: 0.31 kcal/mol	Delta G 0.31 kcal/mol is very low.
16S rRNA -F with	5'-CAATCAGCGTCAGTAATGTTC-3' |||| ::: : 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -4.89 kcal/mol	Delta G -4.89 kcal/mol lie within accepted limit

	5'-CAATCAGCGTCAGTAATGTTC-3' : : : ||||| :: 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -2.64 kcal/mol	Both primers anneal to each other with impossible extension manner.
	5'-CAATCAGCGTCAGTAATGTTC-3' : :: |||| 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -2.03 kcal/mol	Delta G -4.89 kcal/mol lie within accepted limit
	5'-CAATCAGCGTCAGTAATGTTC-3' : : ||| : 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -1.53 kcal/mol	Both primers anneal to each other with impossible extension manner.
	5'-CAATCAGCGTCAGTAATGTTC-3' ||| 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -0.07 kcal/mol	Delta G 0.31 kcal/mol is very low.
16S rRNA-R with *glm*M- F	5'-CTAAGAGATCAGCCTATGTCC-3' : |||| : 3'-TTTGGGGATTGTGGGGATTTTCGAA-5' dG: -2.42 kcal/mol	Delta G -4.89 kcal/mol lie within accepted limit
	5'-CTAAGAGATCAGCCTATGTCC-3' : |||| :: 3'-TTTGGGGATTGTGGGGATTTTCGAA-5' dG: -2.42 kcal/mol	Delta G -4.89 kcal/mol lie within accepted limit
	5'-CTAAGAGATCAGCCTATGTCC-3' : : ||| : 3'-TTTGGGGATTGTGGGGATTTTCGAA-5' dG: -1.53 kcal/mol	Both primers anneal to each other with impossible extension manner.
	5'-CTAAGAGATCAGCCTATGTCC-3' |||| :: : 3'-TTTGGGGATTGTGGGGATTTTCGAA-5' dG: -1.30 kcal/mol	Both primers anneal to each other with impossible extension manner.
	5'-CTAAGAGATCAGCCTATGTCC-3' |||| : : : 3'-TTTGGGGATTGTGGGGATTTTCGAA-5' dG: -1.30 kcal/mol	Both primers anneal to each other with impossible extension

		manner.
	5'-CTAAGAGATCAGCCTATGTCC-3' ||| 3'-TTTGGGGATTGTGGGGATTTTCGAA-5' dG: -0.33 kcal/mol	Both primers anneal to each other with impossible extension manner.
16S rRNA-R with *glm*M- R	5'-CTAAGAGATCAGCCTATGTCC-3' :: : : ||| : 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -1.53 kcal/mol	Both primers anneal to each other with impossible extension manner.
	5'-CTAAGAGATCAGCCTATGTCC-3' |||| 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -1.30 kcal/mol	Both primers anneal to each other with impossible extension manner.
	5'-CTAAGAGATCAGCCTATGTCC-3' ||| : : 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -0.33 kcal/mol	Both primers anneal to each other with impossible extension manner.
	5'-CTAAGAGATCAGCCTATGTCC-3' : ||| 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -0.08 kcal/mol	Delta G -0.08kcal/mol is very low.
	5'-CTAAGAGATCAGCCTATGTCC-3' : ||| : 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: 0.04 kcal/mol	Both primers anneal to each other with impossible extension manner.
	5'-CTAAGAGATCAGCCTATGTCC-3' : ||| : : :: 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: 0.04 kcal/mol	Both primers anneal to each other with impossible extension manner
***glm*M- F with *glm*M- R**	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' |||||| 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -7.02 kcal/mol	Both primers anneal to each other with impossible extension manner

	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' ||||||| : 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -5.94 kcal/mol	Delta G -5.94 kcal/mol lie within accepted limit
	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' : ||||| : : 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -2.64 kcal/mol	Delta G -2.64 kcal/mol lie within accepted limit
	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' : |||| 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -1.30 kcal/mol	Both primers anneal to each other with impossible extension manner
	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' |||| : : : : 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -1.30 kcal/mol	Delta G -5.94 kcal/mol lie within accepted limit
	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' ||| 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -0.33 kcal/mol	Both primers anneal to each other with impossible extension manner
	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' : ||| 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -0.07 kcal/mol	Delta G -0.07 kcal/mol lie within accepted limit
	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' : : ||| 3'-CGCAATCACAATCTTTCATTCGAA-5' dG: -0.07 kcal/mol	Delta G -0.07 kcal/mol lie within accepted limit

Table (4) Summarizing dimerization strength possibilities.

No.	Possible dimerizations	Annealing strength possibilities (Delta G kcal/mol)	Maximum annealing strength
1	16S rRNA-F - 16S rRNA-R	0.16	0.16
2	16S rRNA-F - *glm*M-F	-1.53, 0.31	-1.53
3	16S rRNA-F - *glm*M-R	-0.07, -4.89 , -2.64 , -2.03 , -1.53	-0.07
4	16S rRNA-R - *glm*M-F	-2.42 , -1.53 , -1.30 , -0.33	-2.42
5	16S rRNA-R - *glm*M-R	-1.53 , -1.30 , -0.33 , -0.08 , 0.04	-1.53
6	*glm*M - F - *glm*M-R	-7.02 , -5.94 , -2.64 , -1.30 , -1.30 , -0.33 , -0.07	-7.02

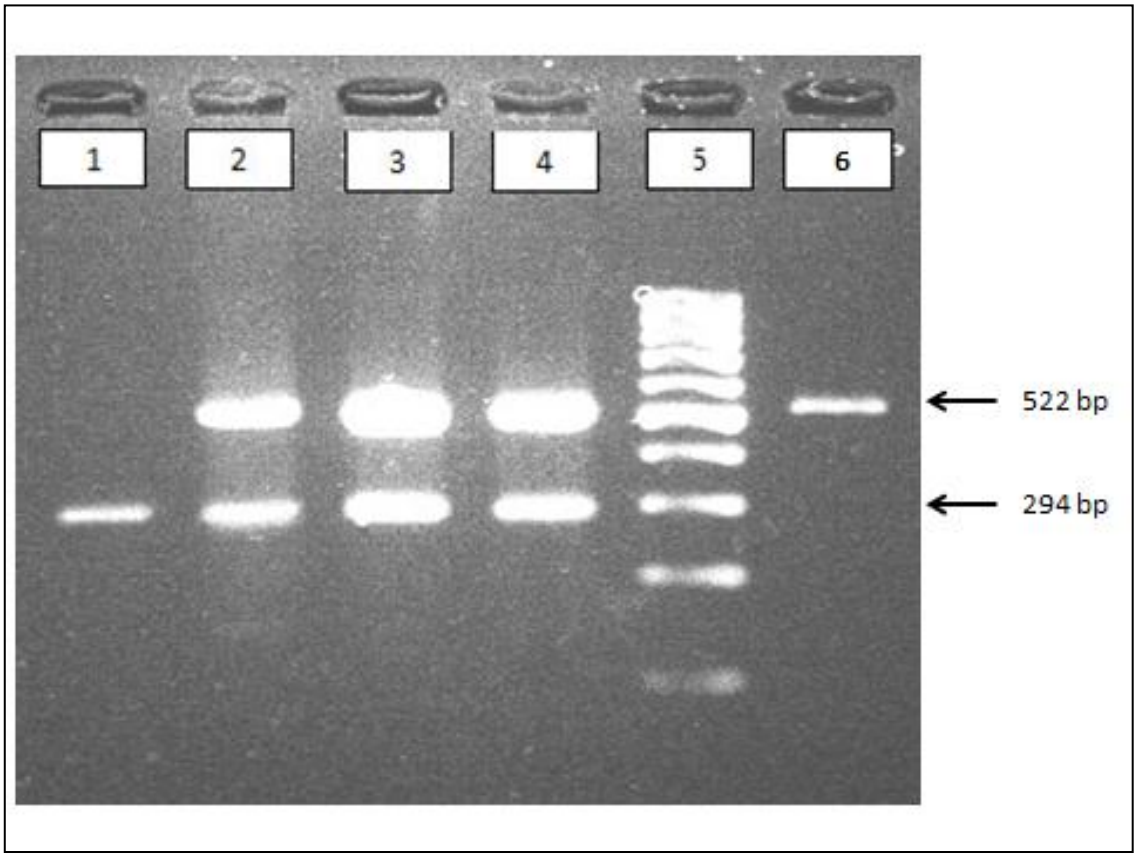

Figure 1: Agarose gel electrophoresis for 16S rRNA and *glm*M genes. Lanes 1= 294 bp of *glm*M gene, 2, 3 and 4= duplex 16S rRNA, 522 bp and *glm*M, 294 bp, 5=100 bp ladder, 6= 16S rRNA, 522 bp.

Discussion

Although PCR method regarded as a very sensitive method for pathogens detection like *H. pylori* (8), but the variation among sequences of the same genes from several strains of the same genus and species of the organism may affect results significantly.

These variations in nucleotide sequences may occur in primers annealing sites, so that they lead to inactivate PCR for positive samples (false negative), or lowering PCR product quantity (weaken PCR).As the results of some researchers indicated several variations in 16S rRNA gene (which used for *H. pylori* detection) from several strains of these bacteria (25).

And because of difficulties to know and predict all sequence variations in bacterial target sequences around the globe, so that to overcome these problems primers designed to anneal with conserved regions in target sequences and to confirm pathogen detection, a duplex PCR for backbone genes is one of the important solutions.

Several researchers designed and commercial kits manufactured a duplex PCR principle based kits (primers sequences are unknown), to gain maximum accuracy as they can by using several backbone genes of *H. pylori*. In addition to accuracy, using duplex PCR saves money, time and efforts. Since that hard works done to find most appropriate primers are continuous to find the optimum combinations.

In addition, some of manufactured kits utilize only 16S rRNA gene, where another kits designated to use several genes like *glm*M, *ure*A, and flagellin (3).

The target of this work was to find a novel combination of primers for detection of both 16S rRNA and *glm*M genes that each one of them previously designed by other researchers and their efficiency improved individually in detecting *H. pylori*. So 15 described primers designated for 16S rRNA detection, and three primers designated for *glm*M gene detection analyzed for duplexing efficiency for the mentioned genes.

The selected primers showed a high efficiency for duplexing according to annealing temperature (55ºC) (6, 22), PCR product sizes (522 bp, 294 bp), in addition to these important factors they make an ideal value for dimerization potential (delta G don't exceed -7 kcal/mol), making the PCR inactivation by controlling the reaction into dimer extension only impossible.

In current work, selected primers for duplexing have dimerization tendency values (deltaG) indicated no theoretically accepted highly strong (primer-primer) annealing either self or hetero dimerization, ranging from +0.16 to -7.02 kcal/mol as indicated in Table 4, except (*glm*M-F with *glm*M-R) with delta G -7.02 kcal/mol which is slightly larger than threshold delta G -7.00 kcal/mol, but the dimerization possibility was with an impossible direction for primer extension (3` to 5`) by top polymerase enzyme. So this form of dimerization doesn't affect PCR. These results in addition to temperature of annealing and PCR product size make the selected primers ideal for duplexing.

However, as indicated by other research, a special attention to primer design parameters such as homology of primers with their target nucleic acid sequences, their length, the GC content, and their concentration needed with duplexing experiments (26). The *glm*M amplification primer in current study needs concentration greater than

16S rRNA primer due to length difference, that *glm*M primers taller than 16S rRNA primer, as indicated in PCR parameter, but individual reaction for each of them separately require the same concentration of each of them, so that the competition on reaction resources must be balanced to gain similar bands volume on electrophoresis.

The primers analyzed in this study to detect 16S rRNA and *glm*M genes have a highly species specific probing for the detection of *H. pylori* with high specificity and sensitivity as indicated by their inventors (6, 22), so their combination must increase these values, and due to cultural and immunological methods difficulties, the direct PCR of biopsy samples will give this method a great importance in detection and subsequent eradication follow up of *H pylori*.

The study concluded that the detection of one of selected genes is enough to indicate the presence of *H. pylori*, however, in case of rarely failure due to strain variability; this could be compensating by the other one.

References

1- Cover, L., Gut, 9 (1) 302142, (2012).

2- Cirak,Y., Akyn,Y. and Mégraud, F., Helicobacter , 12 (1) 4, (2007).

3- Espinoza, C., Vazquez, G. Mendez, M. Vargas, R and Cerezo, G., J., Clin., Microbiol., 49 (4)1650, (2011).

4- Lage, P., Godfroid, E., Fauconnier, A., Burette, A., Butzler, J., Bollen, A and Glupczynski, Y., J., Clin., Microbiol., 33(10) 2752, (1995).

5- Chavali1, S., Mahajan, A., Tabassum, R., Maiti, S. and Bharadwaj, D., a critical examination of predictions, 21(20) 3918, (2005).

6- Engstrand,L., Anne-Marie, H., David, Y., and Fouad A., J., Clin. Microbiol., 30 (9) 2295, (1992).

7- Peek, JR., Miller, G., Tham, T., Pe´rez, I., Cover, L., Atherton, C., Dunn, G., and Blaser, J., J., Clin. Microbiol., 33(1) 28, (1995).

8- Xu, M., Ji, H., Li, S., Man, H., Zhang , F., China. Nati. J. New Gastroenterol, 3(2) 98, (1997).

9- Sonny, K., Chong,F., Qinyuan, L., Fitzgerald, F., and Lee, H., J. Clin. Microbiol., 34(11) 2728, (1996).

10- Tiwari, K., Khan, A., Ibrahim, M., Habeeb, A., Pril , H., World. J. Gastroenterol., 12(14) 2181, (2006).

11- Riggio, P., and Lennon, A., J. Med. Microbiol., 48 (3)17, (1999).

12- Huang, Y., Fan, G., Wang, M., Zhou, H., Tian, F., Li, N., J. Clin. Pathol., 57(1)1273, (2004).

13- Silva , G., Tinoco, M., Rocha, A., Camargos Rocha, M., Guerra, B., Saraiva, E., Queiroz , M., Mem. Inst. Oswaldo. Cruz, Rio de Janeiro, 105 (5) 657, (2010).

14- Fukuda, K., Kuroki, T., Tajima, Y., Tsuneoka, N., Kitajima, T.,Matsuzaki, S., Furui, J., and Kanematsu, T., Carcinogenesis 23(11) 1927, (2002).

15- Sasaki, T., Hirai, I., Izurieta, R., Kwa, H., Estevez, E., Saldana, A., Calzada, J., Fujimoto, S., Yamamoto, Y., Science, j., 40 (7) 413, (2009).

16- Tiwari, K., Khan, A., Ahmed, S., Ahmed, I., Kauser, F., Hussain, A., Ali, M., Alvi, A., Habeeb, A., Abid, Z Ahmed, N., Habibullah, M., Singapore , Med. J. , 46 (5) 224 , (2005).

17- Lu, J., Perng, L., Shyu, Y., Chen, H., Lou, Q., Chong, F., And Lee, H., J. Clin. Microbiol , 37 (3) 772, (1999).

18 - Qiu, B., Zhang, Y., Keshari,P., Wang, Q., Zhou, W. , Xu, Z., , Wang, W., Zhan,Q., Li, W, B.M.C. Cancer, 10(1)374 , (2010).

19- Fernando, N., Holton, J., Vaira, D., DeSilva, M. and Fernando, D., J. Clin. Microbiol., 40(7) 2675, (2002).

20- Milyani, R., Journal of American Science, 7(6)136, (2011).

21- Bickley, J., Owen, J., Fraser, G., and Pounder, E., J. Med. Microbiol. 39(1)338, (1993).

22- Lim, Y., Lee, H., Cho, J., Chang, W., Kim, Y., Myong, H., Lee, K., Rhee, H., and Kook , H., J., Clin., Microbiol., 41(7) 3387, (2003).

23- Peña, A., McNeil, K., Fox, G., and Versalovic, J., J., Clin., Microbiol., 40(4)1511, (2002).

24-Smith, I., Fowora1, A., Otegbayo, A., Abdulkareem, B., Emmanuel A. Int., J., Mol. Epidemiol. Genet., 2(2)178, (2011).

25- Bruce, W., ECKLOFF, Podzorski, P., KLINE, C., and Cockerill , R., Int. J. Sys. Bacteriol. , 44 (2) 320 (1994).

26- Elnifro, M., Ashshi, M., Cooper, J and Klapper, E. Clin. Microbiol. Rev., 13 (4) 559, (2000).