European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 7, Issue 07 , 2020

GENOTYPING OF VAC A GENE IN HELICOBACTER PYLORI

Ms. Sajmi Sathar[1], Dr. Anand Thirupathi[2]

[1]Research Scholar, SRK University, Bhopal, India.

[2]Research Supervisor, SRK University, Bhopal, India.

ABSTRACT

The aim of this study was to identify the presence of H. pylori in biopsy specimens from symptomatic patients by PCR. In addition, the rate of vacA virulence genes was determined. 194 antral gastric biopsy specimens were collected during endoscopy from patients suffering from gastroduodenal symptoms. The samples were collected by the gastroenterologists in their own clinics in. DNA was extracted from the biopsies and subsequently used for PCR identification of H. pylori and the virulence genes using specific primers. The rate of positive H. pylori in the collected biopsies was 44%. The rates of the virulence genes in this sample: vacA was vacAs, vacAi and vacAm respectively. Much research is necessary to determine the presence of an association of this gene with gastric pathology. Variation in the rates of the vacA gene in different countries is a strong indication of its geographical distribution. This study would provide important information regarding the prevalence of virulence genes (vacA) in H. pylori strains in the sample tested in this country.

Keywords: H.pylori, Culture, vacA gene, PCR.

INTRODUCTION

Helicobacter pylori is a microaerophilic, spiral shaped Gramnegative bacterium that colonizes the human stomach. It has been linked to chronic active gastritis, peptic ulcers disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma (Marshall and Windsor, 2005). H. pylori has been classified as a definite class I carcinogen by the World Health Organization (Palli et al.,2007;Versaloic, 2003Although prevalence of H. pylori infectionmay exceed 70% in some countries (Sarari et al., 2008), only a small percentage of the population develop severe disease. This can be attributed to the involvement of specific factors that contribute to the pathogenicity of this organism. The vacuolating cytotoxin (vacA) gene encodes for the vacuolating cytotoxin, the pore forming toxin which causes progressive vacuolation and injury to gastric epithelium (Brito et al., 2003). The induced by contact with epithelium (iceA) A gene has been considered as the marker for peptic ulcer disease.

The aims of this study were to identify H. pylori directly from biopsy specimen collected from symptomatic patients using primers to amplify the vacA genes and to determine the rate of virulence genes, vacAs, vacAi and vacAm , in the biopsy samples by PCR.

MATERIALS AND METHODS

Specimen Collection and Processing

Antral gastric biopsy specimens were collected during upper endoscopy from 194 patients suffering from gastroduodenal symptoms. Patient's consent to participate in this study was obtained prior to enrollment. The samples were collected by gastroenterologists.DNA was extracted from the biopsies by the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Extracted DNA was used for subsequent PCR experiments.

Polymerase Chain Reaction

PCR analysis of the targeted genes was performed using Thermostat Taq DNA polymerase (ABgene, UK) and manufacturer-provided reaction buffer. Five microlitre of DNA were added to 50µl of reaction mixture containing 1×PCR buffer, 1.5mM MgCl2, 0.2mM (each) dNTPs (ABgene, UK) and 0.5µM of respective oligonucleotide primers. 1.25U/µl thermostat Taq DNA polymerase (ABgene, UK) was added to each tube. PCR was performed with a thermal cycler (MJ Research, USA). The amplification cycles consisted of an initial denaturation of target DNA at 95°C for 15min and then denaturation at 94°C for 1 min, primer annealing at 60°C for vacA respectively for 1 min and extension at 72°C for 1 min. All reactions were performed through (35 cycles). The final cycle included an extension step for 5 min. The primers used to amplify the targeted genes (Table-1). Negative controls were added to each PCR run including all reagents except template DNA which was substituted with ultra pure water (Sigma-Aldrich, UK). Amplification of DNA was analysed by agarose gel electrophoresis using standard procedures (Van Doorn et al., 1998).

Mutational analysis

DNA sequencing was carried out by using a Big Dye Terminator DNA sequencing kit v3.1 (Applied Biosystems, UK). Direct sequencing on chromosomal DNA was done with 2µl of chromosomal DNA, 0.25µl of primer (10pmol per µl), 2µl of Big Dye buffer and 2µl of Big Dye under the following conditions: a denaturation at 96°C for 10 s, annealing at 50°C for 20 seconds, and extension at 60°C for 4 min over 30 cycles, followed by Agencourt Clean Seq cleanup and analysed with ABI automated sequencer 3130xl machine (Applied Biosystems, UK). DNA sequence editing and analysis was performed with the programs Bioedit and EMBL-EBI- Clustalw. Sequences were aligned using DNAMAN.

Antibacterial Activity of Liposomal fatty acid against Helicobacter pylori

To determine the antimicrobial activity of LipoFFAs against H. pylori, a bacterial pellet was collected from an overnight liquid culture of H. pylori by centrifugation (3000 × g for 10 min) and resuspended in fresh BHI. The concentration of bacteria was measured by optical density at 600 nm (OD600), OD600 of 1.0 corresponding to approximately 1×108 colony forming units (CFU)/mL. H. pylori (1×107 CFU in 200 µL) were incubated with various concentrations of of LipoFFA ranging from 62.5 to 1000 µg/mL in a 96-well plate at 37°C under microaerobic conditions on a reciprocal shaker for 30 min. For time-dependent antibacterial activity of LipoLLA, bacteria were incubated with 200, 300, or 400 µg/mL of LipoLLA for 5,

10, 20, or 30 min. After incubation, the samples were centrifuged (3000 × g) for 10 min and washed twice with PBS to remove residual LipoFFA.

The bacterial pellet was resuspended in PBS, followed by a series of 10-fold dilutions and the bacterial suspension inoculated onto Columbia agar plates supplemented with 5% laked horse blood. The agar plates were incubated for 4 days before counting colonies.

Outer membrane permeability assay

The outer membrane permeabilization was assessed by measuring the uptake of NPN (Loh et al., 1984). An overnight culture of H. pylori was harvested, washed and adjusted to an OD600 of 1.0 as described above. Approximately 5×106 bacterial cells were treated with 400 μg/mL of LipoLLA for 5 min. Bare liposome and PBS were used as negative controls.

After treatment, each bacterial suspension was centrifuged and resuspended in 200 μL of PBS. For NPN assay, 50 μL of NPN (20 mM final concentration) were added and mixed with treated bacteria. Fluorescence measurements were taken after shaking for 3 min, using a SPECTRA max GEMINI EM microplate reader (Molecular Devices, Inc., Sunnyvale, CA) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The NPN assays were performed at room temperature, repeated three times and the results expressed as relative fluorescence units (Helander and Mattila-Sandholm, 2000).

Plasma membrane permeability assay

Release of ATP from bacterial cells was measured by BacTiter-Glo microbial cell viability assay kit (Promega Inc.) as an indicator of plasma membrane permeability (Parsons et al., 2012). H.pylori was treated with 400 μg/mL of LipoLLA or bare liposome for 5 min. One-milliliter from each sample containing approximately 1×106 bacterial cells was centrifuged at 14,000 × g for 5 min and the supernatant containing released ATP from treated bacteria collected. BacTiter-Glo reagent (Promega Inc.) was added directly to the supernatants in each well of a 96-well plate at a ratio of 1:1. The mixture was shaken for 2 min and luminescence measured on a SPECTRA max M2e microplate reader (Molecular Devices, Inc.).

Transmission Electron Microscopy (TEM)

The effects of LipoLLA on the structure of H. pylori were examined with TEM. Briefly, an overnight culture of H. pylori was treated with LipoLLA (final concentration 95 -400 μg/mL) and incubated for 5 or 30 min. In addition, H. pylori were treated with 200 μg/mL of LipoLLA for 30 min. PBS was used as negative control. Samples were centrifuged at 3000 × g for 5 min and bacterial pellets fixed by resuspending in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Bacterial pellets were then embedded in 2% agarose, post-fixed with 1% osmium tetroxide, and processed in Durcupan resin (Sigma-Aldrich). Fifty-five nm sections were examined using a FEI Tecnai G2 transmission electron microscope equipped with a digital camera.

Scanning Electron Microscopy (SEM)

Morphological changes of H. pylori treated with LipoLLA was observed by SEM. H. pylori was incubated with 400 μg/mL LipoLLA for 5 min and the bacteria harvested and

processed for visualization by an FEIXL30 Environmental SEM. Bacteria were prepared for SEM as previously described (Obonyo et al., 2012). Briefly, untreated and treated bacteria were centrifuged to remove the supernatant, and the remaining pellet was fixed with 2% glutaraldehyde for 2 hr at room temperature. Post fixing, the sample was centrifuged to remove glutaraldehyde and resuspended in 100 μL water. A bacterial suspension was spotted onto a polished silicon wafer and allowed to dry overnight in a biosafety cabinet. The samples were then coated with chromium before SEM imaging.

The biopsy specimens of the patients are processed and standard tests done for diagnosis of H. pylori. The tests are direct smear, rapid urease and culture. Out of the 194 patients tested, direct smear positive are 140, rapid urease positive are 54 and 121 are culture positive. Anaerobic jar failure resulted in the loss of 12 cultures. Another 17 cultures could not be recovered after storage at -80°C, which left a total of 121 primary cultures available for genotyping studies.

RESULTS

H. pylori culture positive gastric biopsies

The biopsy specimens of the patients are processed and standard tests done for diagnosis of H. pylori are shown in Fig -1. The tests are direct smear, rapid urease and culture. Out of the 194 patients tested, direct smear positive are 140, rapid urease positive are 54 and 121 are culture positive. Anaerobic jar failure resulted in the loss of 12 cultures. Another 17 cultures could not be recovered after storage at -80°C, which left a total of 121 primary cultures available for genotyping studies.

Genotypic features of H.pylori

Most of the samples were positive for vacA at least for one of the alleles (the s-region or the m-region) (Table-2). The most virulent vacA s1 allele reported in most studies was predominantly present in these strains 58/100 (58%), and was visualised as a band of 259bp on agarose gel electrophoresis, whereas 26/100 (26%) of the isolates had the vacA S2 genotype .Combined vacA s1/s2 was detected in 16 (16%) of the strains (Fig-2).

The middle region of the vacA gene was detected in 94/100 (94%) of the strains. vacA m1 was detected in 8 (8%) strains while m2 was found in 50 (50%) of the strains. Combined vacA m1/m2 was detected in 36 (36%) of the strains. The s genotypes were more equally distributed than m1(Fig-4) and m2 (Fig-5) genotypes.

Genotyping of the middle region failed in 6/100 (6%) of the strains. s1m2 and s2m2 genotype were the most common allelic combinations of the vacA gene among the strains. Eight percent of the strains harboured s1m1 genotype and amplicons were detected for vacA i1 and i2 (Fig-3) when typed for vacA i and so those cultures were identified as having both types. Six different vacA types were detected; s1/i1/m1, s1/i1/m2, s2/i1/m2, s1/i2/m2, s2/i2/m2, and s1/i2/m1. The genotype s2m1 was not identified in this study.

Multiple vacA genotypes were detected in this study 46/100 (46%) with vacA s1m1m2 genotype being the most prevalent allelic combination 28(28%) indicating the presence of mixed

infection. Detection of m1 and m2 alleles of the vacA gene. Multiple vacA genotypes appeared to be more prevalent in patients with non ulcer dyspepsia (20/40, 50%) than in those with the other disease conditions enrolled in this study, although the difference was not statistically significant ($p>0.05$).

Bactericidal activity of LipoLLA against H. pylori

Antibacterial activities of LipoFFA, including LipoLLA against H. pylori were evaluated in vitro by determining their minimal bactericidal concentrations (MBC) (Fig-1). MBC for this study was defined as the minimal concentration that kills 99.99% (4 log) of targeted bacteria during 30 min incubation.

Accordingly, the MBC value of LipoLLA was determined to be 200 μg/mL, where it killed 99.99% (~4 log) of H. pylori. When the LipoLLA concentration reached 400 μg/mL, no viable bacteria were detected.

Effect of LipoFFA on the outer membrane of H. pylori

The NPN assay was used to determine the outer membrane permeability of H. pylori in response to lipoFFA treatment. There was no difference in NPN uptake in LipoLLA-treated H. pylori compared to the PBS control or bare liposome. H. pylori treated with LipoLLA had a significant ($P < 0.001$) increase in the fluorescence signal of NPN compared to PBS control, indicating that LipoLLA increased the outer membrane permeability of H. pylori (Fig- 6).

Effect of LipoFFA on the plasma membrane of H. pylori

The release of ATP from the cytoplasm through the plasma membrane of lipoFFA-treated bacteria as an indicator of plasma membrane disruption. H. pylori treated with LipoLLA released ATP at the same level as bacterial cell incubated with bare liposomes or control PBS. Treatment with LipoLLA or significantly ($P<0.01$) increased ATP release from H. pylori compare to control.When the effects of the LipoLLA treatment resulted in significantly ($P < 0.01$) greater release of ATP.

LipoFFA-induced morphological changes

Morphological changes of H. pylori when treated with LipoLLA for 5 min by TEM and compared the SEM images with at higher magnification. The outer membrane of the untreated H. pylori adhered closely to the plasma membrane and enclosed a relatively homogeneous cytoplasm. Their cell surface appeared smooth and homogenous when examined under SEM. For LipoLLA-treated H. pylori, TEM images showed membrane detachments between the outer and plasma membrane accompanied with leakage of cytoplasmic contents into the intermembrane space. Concomitantly, SEM images displayed destructive outline caused by blistering of the outer membrane.

DISCUSSION AND CONCLUSION

According to the results of this study, the most virulent vacAs1 allele was predominant in this study population 58(58%) finding which has also been observed in other studies in South Africa and the world (Sugimoto et al., 2009). Brooks et al. (2004) reported a high prevalence of vacAs1 H.pylori strains in their study in South Africa. HoandH.M.Windsor (2000) also

demonstrated a high prevalence of vacAs1 in their study in Ethiopia and Nigeria respectively. These results are however contrary to the findings noted in African Arabs who are predominantly infected with the s2 type allele (Ar´evalo-Galvis et al., 2012).

Meanwhile, s1m2 and s2m2 were the most common combinations of the vacA gene in this study population . The genotype s1m1 was detected in 8% of all the strains analysed. These results are in accordance with the findings of Proenc¸a-Modena,et al. (2009) that delineated a high prevalence of s1m2 and a low prevalence of s1m1. However, the frequency of vacA s1m1 allelic type in this study is lower than those reported from the Netherlands (36%), Hong Kong (26 to 31%) and Nigeria (24%) (M´egraud and P. Lehours, 2007). This may be a reflection of the great heterogeneity exhibited by this organism.

In this study, the prevalence of vacAm2 (50%) was higher than vacAm1 (8%).The results are similar to the findings of Yamaokag et al. (1999) who reported a higher prevalence of m2 in their study; however, some authors have documented a higher prevalence of m1 in their study area .

This finding is in line with several studies in different parts of the world but contrary to the finding of Asrat et al. (2004) and Smith et al. (2002) who reported the presence of this allele though in low percentages; 2% and 6.7% respectively in their various investigations. However, the fact that the s2m1 allele was not found in this study cannot be completely ruled out for we detected multiple vacA genotypes (s2m1m2) of which s2m1 was a makeup. The allele s2m1 has been noted to suffer from a selective disadvantage (Yakoob et al., 2009).

In the present study, LipoLLA increased the outer membrane permeability of H. pylori but had weak effects on the permeability of the plasma membrane and hence poor killing of H. pylori. Potency of FFAs may be influenced by the FA present in lipopolysaccharides (LPS) and phospholipids of the membrane. While unsaturated FAs were not detected in the LPS of the outer membrane, some of them including OA were present in small amount in phospholipids of H. pylori, which may affect the antibacterial activity of OA. Indeed SA, which is known as one of the major fatty acids in LPS of H. pylori (Keshavarz et al., 2009), had no effect on membrane permeability or growth of H. pylori. In last, LipoLLA is a potent bactericidal agent against H. pylori that permeabilizes and disrupts membranes and acts very rapidly in a dose-dependent manner. Due to this highly destructive mechanism of bacterial killing combined with our successful in vivo delivery system, LipoLLA could be a potential therapeutic agent against H. pylori infection.

In conclusion, this study would provide important information regarding the rate of virulence factors in this country. Determination of virulence genes may provide information regarding the risk of clinical outcomes in symptomatic patients.

REFERENCE

Ar´evalo-Galvis, A. A. Trespalacios-Rangell, W. Otero, M. M. Mercado-Reyes, and R. A. Poutou-Pi˜nales, “Prevalence of cagA, vacA, babA2 and iceA genes in H. pylori from Colombian patients with functional dyspepsia,” Polish Journal of Microbiology, vol. 61, no. 1, pp. 33–40, 2012.

Brooks, D. Ahmed, M. A. McConnell, and G. O. Barbezat, "Diagnosis of Helicobacter pylori infection by polymerase chain reaction: is it worth it?" Diagnostic Microbiology and Infectious Disease, vol. 50, no. 1, pp. 1–5, 2004.

Egraud and P. Lehours, "Helicobacter pylori detection antimicrobial susceptibility testing," Clinical Microbiology Reviews, vol. 20, no. 2, pp. 280–322, 2007

Helander, I.M. and Mattila-Sandholm, T. (2000) Fluorometric assessment of gram-negative bacterial permeabilization. J .Appl .Microbiol., **88**: 213–219

HoandH.M.Windsor, "Accuratediagnosis of Helicobacter pylori: polymerase chain reaction tests,"Gastroenterology Clinics North America, vol. 29, no. 4, pp. 903–915, 2000.

Hussein, M.Mohammadi, Y. Talebkhan et al., "Differences in virulence markers between Helicobacter pylori strains from Iraq and those from Iran: potential importance of regional differences in H. pylori-associated disease," Journal of Clinical Microbiology, vol. 46, no. 5, pp. 1774–1779, 2008.

Keshavarz, A., Izadi, B., Rezaei, M. and Shahkarami, A.(2009). A comparative study of eradication of H. pylori infection in dyspeptic patients using a low dose and a high dose triple therapy with clarithromycin, amoxicillin and Omeprazole. Behbood J. **13**: 20-7.

Loh, B., Grant, C. and Hancock, .R.E. (1984) Use of the fluorescent probe 1-N-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy 26: 546–551.

Marshall and H. M.Windsor, "The relation of Helicobacter pylori to gastric adenocarcinoma and lymphoma: pathophysiology, epidemiology, screening, clinical presentation, treatment, and prevention," Medical Clinics of North America, vol. 89, no. 2, pp. 313–344, 2005.

Obonyo, M., Zhang, L., Thamphiwatana, S. and Pornpattananangkul, D. (2012) Antibacterial activities of liposomal linolenic acids against antibiotic-resistant Helicobacter pylori. Mol. Pharm., **9**: 2677–2685.

Palli, G. Masala, G. Del Giudice et al., "CagA+ Helicobacter pylori infection and gastric cancer risk in the EPIC-EURGAST study," International Journal of Cancer, vol. 120, no. 4, pp. 859– 867, 2007.

Parsons, J.B., Yao, J., Frank, M.W., Jackson, P. and Rock, C.O .(2012). Membrane disruption by antimicrobial fatty acids releases low-molecular-weight proteins from Staphylococcus aureus. J. Bacteriol., **194**: 5294–5304.

Proenc¸a-Modena, G. O. Acrani, and M. Brocchi, "Helicobacter pylori: phenotypes, genotypes and virulence genes," Future Microbiology, vol. 4, no. 2, pp. 223–240, 2009.

Sarari,M.A. Farraj, W. Hamoudi, and T.A. Essawi, "Helicobacter pylori, a causative agent of vitamin B12 deficiency," Journal of Infection in Developing Countries, vol. 2, no. 5, pp. 346–349, 2008.

Shiota, M. Watada, O. Matsunari, S. Iwatani, R. Suzuki, and Y. Yamaoka, "Helicobacter pyloriiceA, clinical outcomes, and correlation with cagA: a meta-analysis," PLoS ONE, vol. 7, no. 1,Article ID e30354, pp. 1–7, 2012.

Sugimoto, J. Y. Wu, S. Abudayyeh et al., "Unreliability ofresults of PCR detection of Helicobacter pylori in clinical orenvironmental samples," Journal of Clinical Microbiology, vol.47, no. 3, pp. 738–742, 2009.

Van Doorn, L–J., Figueiredo, C., Sanna, R., Plaisier, A., Schneeberger, P., De Boer, W. and Quint, W. (1998). Clinical relevance of the cagA, vacA, and iceA status of Helicobacter pylori. Alimentary Tract. **115**(1):58-66.

Versalovic, "Helicobacter pylori: pathology and diagnostic strategies," American Journal of Clinical Pathology, vol. 119, no. 3, pp. 403–412, 2003.

Yakoob, S. Abid, Z.Abbas et al., "Distribution of Helicobacter pylori virulence markers in patients with gastroduodenal diseases in Pakistan," BMC Gastroenterology, vol. 9, article 87, pp. 1–7, 2009.

Yamaoka, T. Kodama, O. Gutierrez, J. G. Kim, K. Kashima, and D. Y. Graham, "Relationship between Helicobacter pyloriiceA, cagA, and vacA status and clinical outcome: studies in four different countries," Journal of Clinical Microbiology, vol. 37, no. 7, pp. 2274–2279, 1999.

Table-1: PCR reaction conditions used for H. pylori genotyping

Gene	Denaturation	Annealing	Extension	N of cycles	Source
	Temperature °C (Time)				
vacA s	94 (1 min)	52 (1 min)	72 (1 min)	35	(26)
vacA i	95 (30 sec)	52 (1 min)	72 (1 min)	35	(29)
vacA m	94 (1 min)	57 (1 min)	72 (1 min)	35	(25)

Table-2: **Genotype distribution of H. pylori cultures**

Genotype	H. pylori cultures	
vacA type	**n**	**% of 121**

s1/i1/m1	27	22
s1/i2/m2	44	36
s2/i2/m2	29	24
s1/i1/m2	1	1
s2/i1/m2	3	2
s1/i2/m1	1	1
s1/i1/m1 + s1/i1/m2	1	1
s2/i1/m2 + s2/i2/m2	2	2
s1/i1/m1 + s1/i2/m1 + s1/i1/m2 + s1/i2/m2	8	7
Presence of vacA alleles hypothesized to be more virulent, with or without other alleles		
s1	82	68
i1*	32	26
m1*	28	23

Fig-1: Positive by standard tests

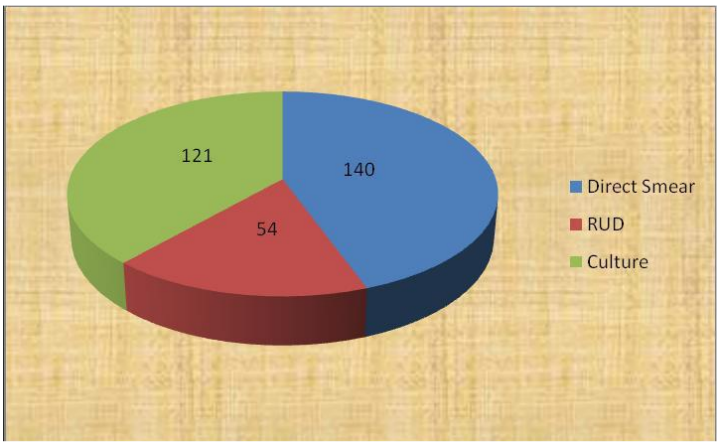
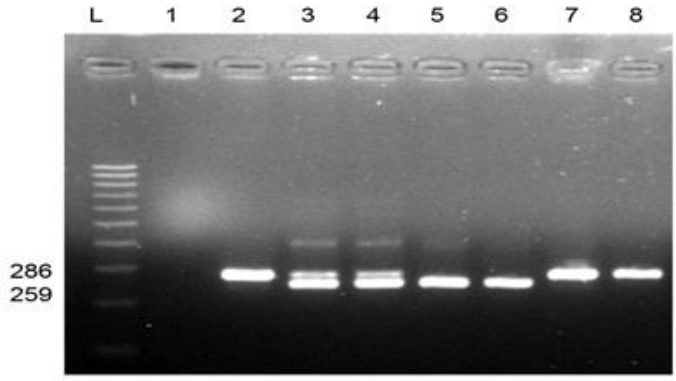
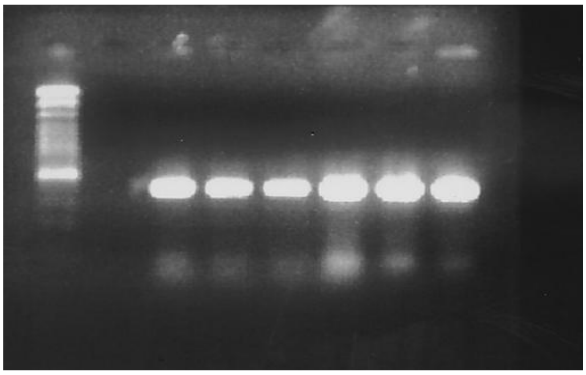


Fig-2: Detection of s1 and s2 alleles of the vacA gene



Lane L, marker; lane1 negative control; lanes 2, 7 and 8 s2 allele; lanes 5 and 6 s1 allele; lanes 3 and 4 s1/s2 alleles
Numbers on the left indicate molecular size (in base pairs).

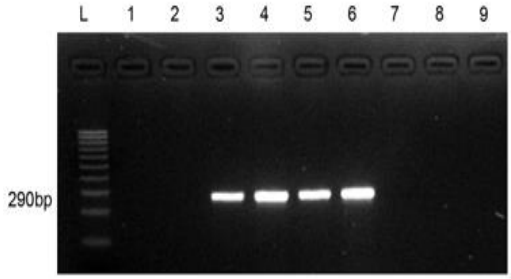
Fig-3: **Detection of i1 and i2 alleles of the vacA gene**
L 1 2 3 4 5 6 7



286 bp

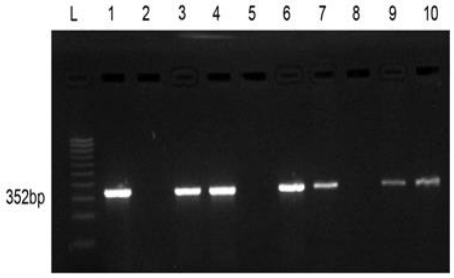
Lane L, marker; lane1 negative control; lanes 2, 7 and 8 i2 allele; lanes 5 and 6 i1 allele; lanes 3 and 4 i1/i2 alleles

Fig-4:Detection of m1 allele of the vacA gene.



Lane L, marker; lanes 1, 2, 7, 8 and 9 m1 absent; lanes 3, 4, 5 and 6 m1 present

Fig-5:Detection of m2 allele of the vacA gene



Lane L, marker; lanes 1, 3, 4, 6, 7, 9 and 10 m2 positive; lanes 2, 5 and 8 m2 negative

Fig-6:Concentration of bactericidal activity of LipoLLA against H.pylori