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Purification and Molecular Cloning of Cytotoxin Associated Gene (Caga) from *Helicobacter Pylori* Strains Isolated from Clinical Samples

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Abstract: *Helicobacter pylori (H. pylori) are spiral, gram negative, microaerophilic bacteria which aids in the pathogenesis of gastric and duodenal cancers. Its vast invasiveness and repeated gaining of antibiotic resistance has become troublesome for the scientific community. These strains harbour the intestinal walls via many anchor proteins and virulence factors. One of such is (cytotoxin associated gene) cagA which is found be the major factor for the pathogen in linking to the intestinal wall. The present study was designed to isolate and purify the cagA gene from the clinical samples and to clone the gene into bacterial strains which can be used in the development of novel peptide vaccines. The cagA gene was purified and cloned into DH5α strain and confirmed for the presence of gene of insert using the restriction enzymes. The study was further planned to express the desired peptide fragments of the cagA protein into an expression vector for designing a novel peptide vaccine.*

Keywords: *Heliocbacter pylori, cagA, PCR, Cloning.*

1. INTRODUCTION

Vaccines are usually developed basing on their attenuation or inactivation of the pathogens. Studies done so far in the field of molecular biology, genomics and proteomics have paved new ways in the vaccine production [1]. The novel use of therapeutic proteins produced due to rDNA technology not only aims at producing novel vaccines but also in targeting the immune responses against the protective antigens. Many of the expression systems have been tried in the production of therapeutic proteins. Live recombinant bacteria can be effectively modified and moreover has more adjuvant properties. DNA vaccines consist of non-replicating plasmids [2] which are found to induce long-term cellular responses through the immune system. They are largely used in the *invitro* or *invivo* systems and they depend on the knowledge of the mode of infection and the counter action by the immune response which is rendered by the host [3].

Helicobacter pylori (*H. pylori*) are the most common pathogen which lives in the human stomach for more than since 60,000 years. This strain was of great significance to the field of bioscience as it was found to be linked to the gastric and peptic ulcers. They are also found to be associated with the gastric lymphoma [4, 1]. *H.pylori* is thought to be involved in the pathogenesis of gastric cancer and its allied diseases. H. pylori eradication was found to be more crucial at the very moment which might rapidly decrease the rate of inflammation within the gastric mucosa [5]. This not only prevents the progression of the disease but also reverses the gastric atrophy before its development into intestinal metaplasia. Many of the factors are involved in *H. pylori* eradication. These include poor patient compliance, internalizing bacteria, massive gastric bacterial loads and high gastric acidity and very most importantly the resistance to antibiotics [6].

Cytotoxicity antigen A (cagA) is the gene which encodes for the protein CagA protein. Basing on its presence the strains are classified into cagA-positive and cagA-negative strains. The CagA protein was found to be between 130 to 145kDa owing to its structural polymorphism within the carboxy-

terminal (C-terminal) domain [9]. As known CagA was considered to be solely responsible bacterial cytotoxin. The cagA was found to be localized at one corner of the cag pathogenicity island (cag PAI). *H. pylori* was thought to adhere to the surface of the gastric epithelial cells and such a adherence is mediated by adhesins like BabA/B, SabA, OipA, HopZ, and AlpA/B [9]. Once they are attached to the gastric epithelial cells *H. pylori* cagA-positive strains initiate the delivery of CagA into the cytoplasm of the host cells.

Molecular studies often confirms of the role of the pathogen in causing a particular disease. Isolation and purification of the strains not only allows us to identify the source of the organism but also the command on the environments the pathogen is having. Molecular characterization studies are paving new ways into finding the molecular and pathophysiologcal mechanisms which will aid in controlling the infection [10]. Epidemiological studies also aid in locating the distribution and moreover will help in determining the vastness and extent of the infection. PCR amplification and RAPD profiling will give the world a chance to find out their close relatives. This not only allows one to find out the possible cause for the spread but also to find out possible antibacterial targets [11].

In principle genetic engineering deals with the ability to manipulate the DNA with precision in invitro conditions. This has led to the new innovative developments within the field of biology and medicine. They led to the discovery of new classes of genes and proteins [Wong, B.C, 2004]. At the same time, they led to the discovery of new and highly conserved proteins. They also led to the prediction of new proteins and their functions [12]. Individual proteins and their domains are estimated of their protein function in both invivo and invitro. Recently studies done on the regulatory regions and their cloning within the fused and unfused genes provided biologists to unravel the complex regulatory networks. Through this process the expression of genes can be controlled [13]. The molecular cloning has paved ways to provide a most practical and effective way to obtain purified molecules. These purified molecules can be sued for a variety purposes. This has led to the discovery of novel therapeutic proteins which eventually led to the increase in the production of biological products. These products are used either in the synthesis of vaccine peptides or designing of novel diagnosis methods [14].

In an attempt to identify, clone, and characterize a new drug target and developing vaccine candidates for the gastric infection by *H.pylori*. The present was designed to isolate and characterize the *H.pylori* from the clinical isolates. These pathogens were identified basing on their morphological and biochemical characterization. cagA gene was isolated and cloned into expression vector.

2. Materials and Methods

Sample Collection

10 samples were collected from Department of Gastroenterology, M.S.Ramaiah Memorial Hospital. The samples were collected from those treated for *H. pylori* infection and those with gastrointestinal bleeding in the last seven to twenty days and processed to the lab for isolation of the *H.pylori* culture. The samples were intended for the rapid urease test for growth and maintenance of *H.pylori*.

Bacterial Isolation

Brain heart infusion agar plates were used for culturing. From the biopsy samples, 10^{-9} and 10^{-10} fold serial dilutions were spread on blood agar plates and incubated overnight at 37^0C. The colonies obtained were used for confirmatory tests Urease test and PCR based tests. The isolated colonies were screened for gram staining and other biochemical characterization tests like catalase, oxidase, and urease. The strains were also confirmed by PCR amplification of a *ure*A fragment.

3. Biochemical Characterization

Catalase Test

Hydrogen peroxide and super oxide when accumulated within the cells leads to the death hence the organisms which processes catalase or peroxidise degrade the oxide radicals enzymatically. The catalase enzyme is thought to neutralize the bactericidal effects of hydrogen peroxide and protects

them. To the loopful of bacterial culture on a clean dry glass slide, a drop of 3% H_2O_2 was added and mixed properly. Appearance of bubbles on the slide indicates positive to the assay.

Oxidase Test

The oxidase test is used to screen those bacteria which can produce cytochrome c oxidase. If the enzyme is present the end product turns purple colour. On absence of the enzyme the reagent remains reduced and is seen colourless. A filter paper soaked in tetramethyl-p-phenylenediamine dihydrochloride was used for the assay and the colony of interest was smeared on the filter paper. The inoculated area turns from deep blue to purple within 10-30 seconds if the strain is positive to oxidase.

Urease

Many of the strains especially those which cause UTIs are said to have urease enzyme which splits urea into ammonia and carbon dioxide. The ammonia combines with carbon dioxide to form alkaline ammonium carbonate which turns the phenol red to bright pink from orange yellow colour. A loopful of test culture was inoculated into the culture medium and incubated at 35°C for 18 to 24 hours. Strains which are positive to urease turn the colour of the slant from light orange to magenta.

Extraction of DNA

Total genomic DNA from the isolated strains were purified by N- Cetyl- N, N, N-trimethyl-ammonium bromide (CTAB) method. The DNA isolation protocol was followed according to Li and Yao. Briefly 1ml bacterial broth culture was centrifuged at about 10000rpm for 2min at 4 °C and the obtained pellet was suspended in 675µl of extraction buffer (100mM TrisHCl, 100mM EDTA, 1.4M NaCl, 1% CTAB and Proteinase K - 0.03µg/µl) and incubated at 37°C for about 30min. The contents are mixed thoroughly and added with 75µl of SDS (20%). Following incubation at 65°C for about 2 hours the contents are mixed thoroughly and centrifuged at 10000rpm at 4°C. The supernatant obtained was added with equal volume of Chloroform: Isoamyl alcohol (24:1) and centrifuged at 10000rpm for 10min at 4°C. The upper aqueous layer was collected and 0.6volumes of isopropyl alcohol was added and incubated for 1hour at RT and centrifuged at 10000rpm. The pellet obtained was washed with 500µl of 70% ethanol and stored until further use.

Amplification of Urea Gene

The ureA gene encodes for urease enzyme and is present exclusively in those UTI strains which can convert urea to ammonia. *Helicobacter pylori* is thought to be positive for urease and hence the study was designed to screen the ureA gene. The specific primers for ureA gene (table 1) were designed using the Primer3 Plus software and ordered through HiMedia.

Table1. *Table showing the details of primers of ureA and cagA gene*

Gene	Primer	Sequences (5'- 3')	GC %	Tm	Length	Product
ureA	FP	GAC ATT GGC GGT AAC AGA AG	50	51.8^0C	20	1710bp
	RP	CTA AGG ATT TAA GGA GCA TCG	43	50.5^0C	21	
cagA	FP	TGAATGACAGCCCTGGCAGCA	48	52.4^0C	21	600bp
	RP	CTGAGATGACAAGCTATGAT	49	60.2^0C	20	

The PCR master mixture of about 25µl reaction was prepared with the dNTPs (2.5mM), and primers (FW &RV; 10picomoles/µl), Taq polymerase (5U) and template DNA (50ng/ µl). The PCR parameters were set up with initial denaturation 94°C for 5min, denaturation of 94°C for 45sec, annealing 55°C for 1min, elongation 72°C for 1min with final elongation of 72°C for 10min. The PCR products obtained were run on 1.5% of agarose gel using 1x TAE buffer.

Amplification of CAG Gene

The cagA gene encodes for cytotoxin associated gene which is known to cause virulence in the *Helicobacter* strains. The specific primers for cagA gene were designed using the Primer3 Plus software and ordered through HiMedia. (Table 1). The PCR master mixture of about 25µl reaction was prepared with the dNTPs (2.5mM), and primers (FW &RV; 10picomoles/µl), Taq polymerase (5U) and template DNA (50ng/ µl). The PCR parameters were set up with initial denaturation 94°C

for 4min, denaturation of 94°C for 45sec, annealing 68°C for 1min, elongation 72°C for 1min with final elongation of 72°C for 5min. The PCR products obtained were run on 1.5% of agarose gel using 1x TAE buffer.

DNA Extraction from Gel

The amplified PCR product run on the agarose gel was extracted to perform the cloning experiment. The amplified fragment was extracted by the following protocol. Briefly 300mg of agarose gel fragment was excised and taken into a fresh 1.5ml centrifuge tube. 650µl Gel Solubilizer (500µl of 4.5M NaI, 0.1% sodium thiosulphate) was added and the contents are incubated for 10min at about 50°C. The dissolved gel was centrifuged at 10000g for 1min and added with 700µl of wash Buffer and centrifuged at 10000g for 1min. The DNA filtrate was now eluted with elution buffer and stored at 4°C, until further use.

Cloning and Ligation of the Caga Gene

The purified PCR product was ligated into pMD19-T cloning vector (Fermentas, USA). The eluted PCR products were ligated separately into the pMD19-T cloning vector (Fermentas, USA). The amount of the PCR product to be used for the cloning as like in 1:1 vector to insert ratio was maintained. 30µl of the ligation reaction was setup in 3:1 molar ratio of insert and vector DNA. 0.52pmol of DNA and 50ng/µl of vector DNA was sued in the ligation mixture. The contents are mixed thoroughly and incubated at RT for about one hour.

Transformation

E. coli strain DH 5-α was used in the present study. DH5α™ is the most widely used *E. coli* strain for the cloning experiments and is used mainly because of its highest transformation efficiency, Blue-white selection and easy plasmid isolation. The competent cells were prepared according to the Sambrook and Russel 1989 lab protocol. The ligated product was mixed with 200µl of prepared competent cells and incubated on ice for 30min. Heat shock treatment was given to the ligation and competent cell mixture at 42°C for about 2min. The contents in the tube were transferred immediately onto ice and incubated for 2-3min. 1ml of LB broth was added to the ligated mixture and used for plating. The ligated and transformed cells were plated onto LB agar plates containing Ampicillin (50mg/ml), 200µl of X- gal and 20µl of IPTG. White colonies containing recombinant clones were selected and streaked on a fresh LB plate containing ampicillin and incubated overnight. This serves as a master plate for each tranformant.

Plasmid Isolation

Plasmids from the transformed clones were isolated according to the alkaline lysis method. Briefly 1.5ml of the culture was centrifuged at 12000rpm at 4°C and the pellet was resuspended in 100µl of ice cold solution I (50mM glucose, 25mMTris-Cl and 10mM EDTA; pH 8.0). To the tube 200µl of solution II (0.2N NaOH, 1% w/v SDS). To the mixture 150µl of ice cold solution III (5M potassium acetate, 12.5 ml of glacial acetic acid) was added and centrifuged at 12000rpm for 5min. The supernatant obtained was added with equal volumes of chloroform: isoamyl alcohol (24:1) and centrifuged at 12000rpm at 4°C. Plasmid DNA was precipitated with 0.6volumes of ice cold isopropanol. The purified plasmids obtained were subjected to restriction digestion using restriction endonucleases (Sigma Aldrich, India) to confirm the clones.

Restriction Doublé Digestion

The plasmid and the DNA of insert are double digested with EcoRI (G/AATTC //CTTAA/G) and Hind III (A/AGCTT //TTCGA/A). To the tubes labelled E,H and control the contents are added accordingly. EcoRI (10U/µl) and Hind III (10U/µl) and test DNA (0.2µg/µl) are used as components. The contents are mixed thoroughly and incubated at 37°C for about 4hours. Following incubation the the samples were loaded on 1% agarose gel and electrophoresed.

4. RESULTS

The samples collected from the Department of Gastroenterology, M.S.Ramaiah Memorial Hospital under the supervision of Dr.Avinash, Associate professor, dept of Gastroenterology were processed to the lab and the *H.pylori* isolates were isolated. The isolates obtained were used for further experimentation. The isolates were revived on BD Brain Heart Infusion (BHI) Agar.

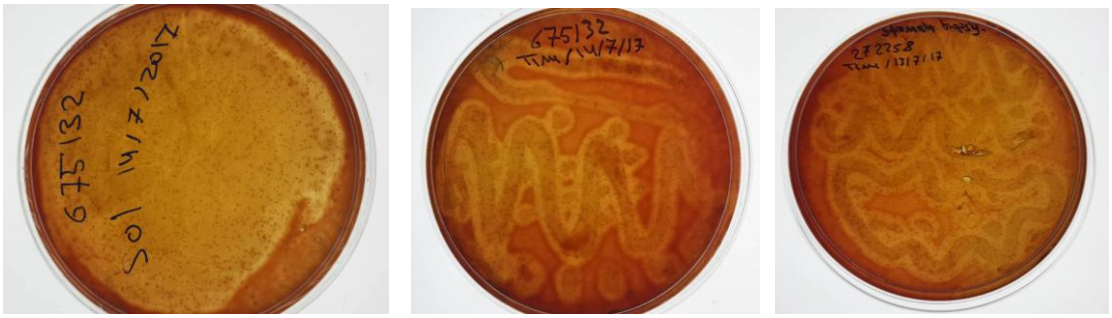

Figure8. *Plate showing the colonies isolated from the clinical samples (Biopsy sample).*

Biochemical Characterization

The given strains were identified to be rod shaped and stained red as saffranine and are considered to be gram negative bacilli.

Assay	Result
Gram staining	Negative
Catalase test	+
Oxidase test	+
Urease test	+

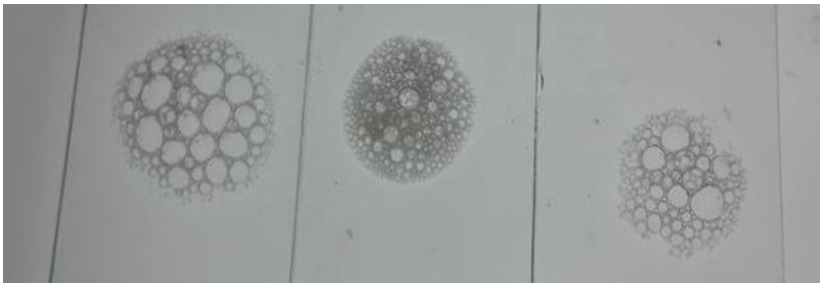

Figure1. *Picture shwoing the results of catalase test done n the isolates*

Figure2. *Picture showing the results of urease test done on the isolates. Top and bottom are the positive isolates and the centre is the negative control.*

Extraction of DNA

Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method. The DNA isolated was stored in refrigerator and used for the quantitative analysis. Among the 54 samples isolated only ten samples whose DNA was found pure was selected for the experiments.

Amplification of Urea Gene

The ten samples obtained from the DNA extraction were amplified using the UreA gene primers. The amplified product was supposed to be 1710bp. All the ten samples showed amplification to the primers. This confirms the strains are positive for the Urease gene. The product was calculated to be approximately 1710bp. This confirmation of the PCR products proves that the samples obtained were of Helicobacter strains.

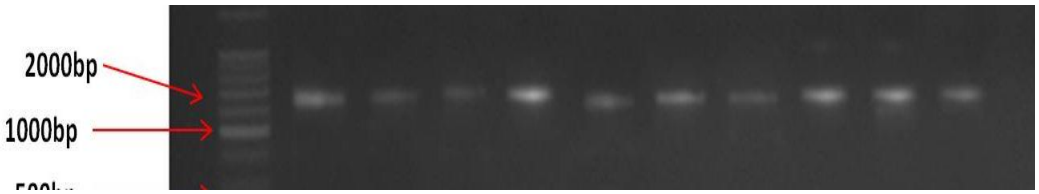

Figure3. *Gel showing the PCR products amplified with UreA gene primers. All the ten samples showed amplification for the gene. Gene product was found to be approximately 1710bp. The gel run was 1.5% agarose gel. Lane M: Marker, 100bp-10000bp marker; Lane 1-Lane 10: DNA samples.*

Amplification of CAG Gene

The PCR product was supposed to be approximately 600bp in length. On running the ten products on the 1.5% agarose gel it was found that all the ten samples showed amplification to the gene. They were positive for the amplification. The calculated size of the gene product was found to be 600bp, which was in accordance with the calculated value and experimental value. Samples 2, 3, and 4 showed more expression than the samples 1 and 5. From the gel photograph it was quite obvious that the expression was not uniform though the amplification was positive for all the five samples. The same result was observed for the remaining 6,7,8,9 and 10 samples. They too showed positive amplification. But 6, 9 and 10 showed more expression than the 7 and 8 samples.

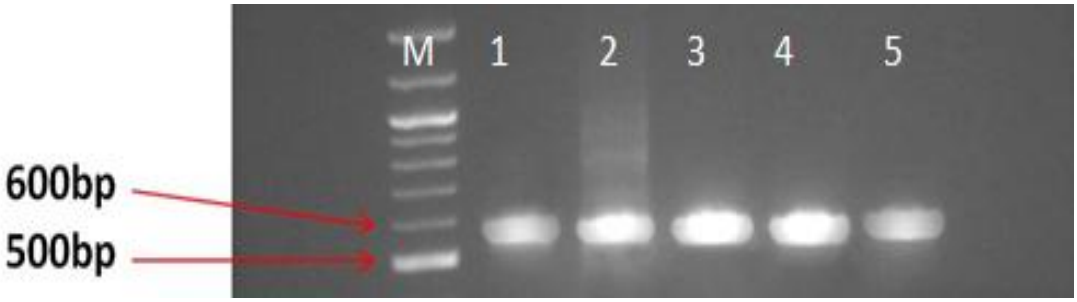

Figure4. *Gel showing the PCR products amplified with cagA gene primers. All the five samples showed amplification for the gene. Gene product was found to be approximately 600bp. The gel run was 1.5% agarose gel. Lane M: Marker, 100bp ladder; Lane 1-Lane 5: DNA samples 1-5.*

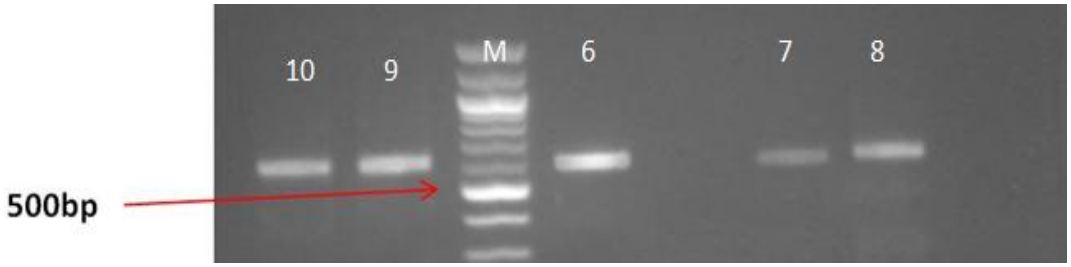

Figure5. *Gel showing the PCR products amplified with cagA gene primers. All the five samples showed amplification for the gene. Gene product was found to be approximately 600bp. The gel run was 1.5% agarose gel. Lane M: Marker, 100bp ladder; Lane 6,7,8,9,10: DNA samples 6,7,8,9 &10.*

Cloning of the Caga Gene

The gene of interest or the amplified product eluted was used in the cloning process using the T vector. The plasmid was found to be 2692bp in size. The ligated product was mixed with competent cells and transformed into bacterial strains. The white colonies were found to be recombinant clones.

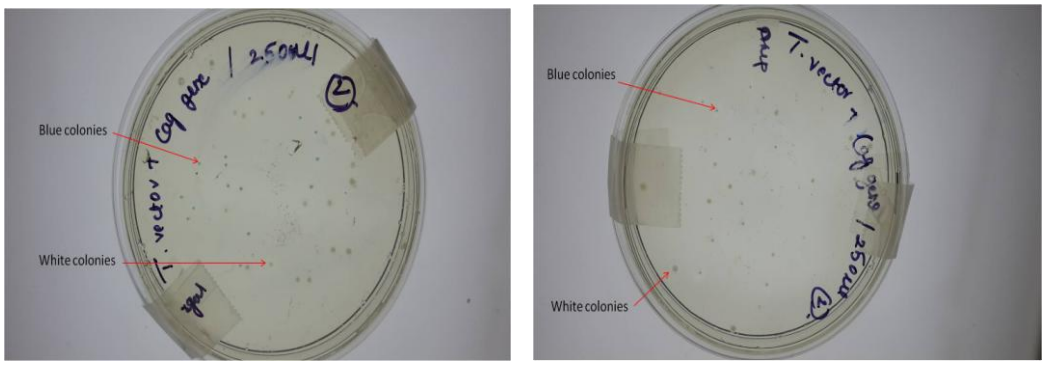

Figure6. *Plate showing the transformed and non transformed colonies. Blue colonies represent the non recombinant; White colonies: Recombinant clones*

The control samples were run in the gel to show the purity of the fragment isolated. The 600bp fragment was in accordance with the size of gene fragment. The HindIII digest was found to be around 250bp and the EcoRI digest was found to be around 350bp. The total again counts to be around 600bp which is again in accordance with the gene of interest.

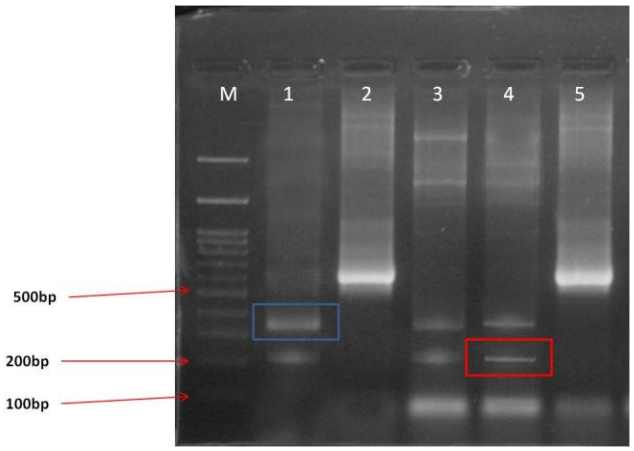

Figure7. *Gel showing the plasmid bands and the digested bands of restriction digestion. The Plasmids isolated from the rDNA clones were double digested with the HindIII and EcoRI. Lane M: marker 100bp, lane 1, 3 & 4: DNA digested; Lane 2, 4: Controls (undigested sample); Blue Box: EcoRI digest; Red box: HindIII digest.*

5. DISCUSSION

Every year, about half a million people get killed due to gastric cancer. The major reason behind the disease is *H. pylori*, a bacterium that colonises the stomach. In more than 20 per cent of those infected, it leads to stomach ulcer and may progress to cancer. To treat *H. pylori* infections, scientists currently use a combination of different antibiotics. However, they do show some serious side-effects and also leads to development of antibiotic resistance. That's the reason why many of the biologists failed to develop a potential vaccine against *H. pylori*.

All of the tissue samples obtained from the hospital were subjected to DNA extraction and the pure DNA samples extracted were further used for the urease enzyme amplification. Among the 54 samples isolated only ten samples whose DNA was found pure was selected for the experiments. The ten samples obtained from the DNA extraction were amplified using the UreA gene primers. The amplified product was supposed to be 1710bp. All the ten samples showed amplification to the primers which confirmed the strain is positive for the Urease gene. The product was calculated to be approximately 1710bp. DNA bands were seen clearly on the gel at about 1700-1800bp. The gene of interest or the amplified product eluted was used in the cloning process and the gene was ligated into

the vector pMD19-T cloning vector. The plasmid was found to be 2692bp in size. The recombinant DNA was then transformed into the competent cells and incubated. The white colonies containing the recombinant clones were selected and streaked on a fresh LB plate containing ampicillin and incubated overnight. Master plate for each transformant was retained. All the colonies selected from the master plate were used for isolating the plasmid DNA and restriction analysis to identify the positive recombinants. Following isolation of the plasmids from the recombinant clones, the gene of interest was isolated from the plasmid using the restriction digestion. The gene of interest isolated was further double digested with two restriction enzymes HindIII and EcoRI.

The control samples run on the gel showed purity of the fragment isolated. The 600bp fragment was in accordance with the size of the gene fragment. The HindIII digest was found to be around 250bp and the EcoRI digest was found to be around 350bp. The total again counts to be around 600bp which is again in accordance with the gene of interest.

All the present studies done confirm of the possible hypothesis that cagA can be sued as peptide vaccine. Peptide vaccines have been proved very effective in terms of potentiality and decreased immune rejection. Such a development would surely aid in the development of new vaccines. Hence my study in isolating and purifying a pathogenic peptide from the protein cagA was successful. That protein was cloned and isolated in its recombinant form. Further experiments need to be designed to isolate and test for the efficacy levels on the patients.

6. CONCLUSION

Numerous studies were done so far to demonstrate the effective protection mechanisms against *H. pylori* infection. The *H. pylori* antigen urease was found to be the first attracted attention for designing a potentially ideal antigen. This was known to be a well-conserved enzyme and is essential for the bacteria to allow the survival of the pathogen within the acidic lumen of the stomach. The extracellular bacterium *H. pylori* also developed very sensitive mechanisms for establishing with the host epithelial cells. They are cagA which encodes approximately 30 genes which are secretory in nature. This also encodes for a pilus-like structure protein which is inserted into the host membrane epithelial cells to transfer the CagA. Hence our study on the cagA protein cloning and expression was successful and might aid in the development of potential candidate for vaccine. The gene of interest was isolated and successfully cloned into the bacterial strain and then cloned to express the protein of interest. The protein isolated was sequenced and designed antibodies for a small peptide. Such antibodies were used for the western blot confirmation. Further studies need to be planned to design proteins of several other peptides and use them for vaccination. Further these peptide fragments need to be tested among the animal models. Once if found the protein of interest was expressed then it can used for the clinical study.

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