CHAPTER 3

RESULTS

The pathogenesis of *H. pylori* in gastro-duodenal diseases is poorly understood. The study carried out in this thesis focuses on the putative role of outer membrane protein that may be involved in the pathogenesis of *H. pylori*. There is strong evidence that the ability of bacterial surface components influence the colonisation and persistence of the pathogen as well as the disease process. Based on such existing reports this study is confined to elucidate the role of outer membrane protein and to question whether there is any difference between clinical strains from different clinical manifestations.

The first section of this work involves the classification and characterisation of the clinical strains using the ATCC strain as the reference strain. Based on this classification few strains were taken to further elucidate the mechanism of pathogenesis. Various cellular fractions from these clinical isolates were used in this study to identify the role played by these fractions in eliciting major responses within the host in a cell-free system. An *in vitro* cell culture system, with HEp-2 cells, a laryngeal carcinoma epithelial cell line was developed as a model to mimic the interactions between *H. pylori* and the gastric epithelium. This model involving the outer membrane protein in a cell free system permits the investigation of the interplay between the bacteria and epithelial cells in a controlled environment. The study showed that the outer membrane protein exerted major morphological, immunomodulatory and toxic effects on the host.
The second part of this thesis elaborates the role of outer membrane protein as a candidate antigen towards the development of immunodiagnostic antigen. In this part of the thesis various antigenic preparations are compared towards identification of a novel antigen in diagnosis.

The last section gives a picture about the existing genotypes for vacA and cagA alleles in Indian clinical isolates. The variation in these genes has been correlated in detail with the different clinical manifestations.

3.1 PRELIMINARY CHARACTERISATION OF CLINICAL STRAINS

3.1.1 Adherence pattern of the clinical strains

Sixteen clinical strains isolated from biopsies of different disease conditions and one reference strain of *H. pylori* were analyzed for their adherence pattern on HEp-2 cells. Microbial adherence was assessed by microscopical evaluation of Giemsa-stained preparation. The various adherence patterns of the clinical isolates are shown in Fig.3.1. Out of this four isolates did not exhibit any adherence (Fig.3.1 KMC 01, MMC 02). Six isolates showed a higher degree of adherence similar to that of the standard strain 26695 (Fig.3.1 26695, KMC 03, MMC 03, and KMC 182). The extent of adherence was lesser for the six strains (Fig.3.1 KMC 04,156). Generally the adherence patterns of these clinical strains were diffused. No internalisation of the bacteria was observed during the coculture of the bacterium with the HEp-2 cells. No correlation was seen between the adherence and disease status of the strain. Our study also confirms that HEp-2, a laryngeal epithelial cell line provides a suitable *in vitro* model for the study of the interactions between *H. pylori* and gastric epithelium (Figueroa et al., 1992). Fig.3.1 shows the representative clinical isolates exhibiting different adherence patterns.
Fig. 3.1 Adherence pattern of clinical strains of *H. pylori* to HEp-2 cells

The extent of adherence and the adherence pattern of the clinical isolates were assessed by observing the Giemsa stained preparation. Representative results of the various adherence pattern exhibited by clinical isolates is shown in this figure. KMC 01 and MMC 02 did not show any adherence. KMC 03 and MMC 03 exhibited a good adherence similar to that of the standard strain 26695. KMC 156 and MMC 04 showed adherence to a lesser extent. Untreated HEp-2 is shown as control.
3.1.2 Characterisation of vacuolating activity of the clinical isolates

Cell-free broth culture supernatants from approximately 50% of *H. pylori* strains induce eukaryotic cell vacuolation *in vitro*, a phenomenon that has been attributed to the cytotoxic activity. The extent of vacuolation induced by the vacuolating cytotoxin in HEp-2 cells is quantitated by neutral red uptake assay (Cover et al., 1993). To assess vacuolating cytotoxin production by clinical strains, culture supernatants from sixteen isolates were incubated with HEp-2 cells for 24 and 48 hours to quantitate vacuolation using the neutral red uptake assay. The HEp-2 cells were then stained with the neutral dye, which occupies the acidic vacuoles caused by *H. pylori* culture supernatants. The neutral red extracted from the vacuoles by acidified alcohol is then quantitated by absorbance at 540nm. Supernatants from all the clinical samples produced vacuolation of cells, but there was difference in the extent of vacuolation of HEp-2 cells. The maximum dilution of supernatants that produced vacuolation was 1:1 and 1:2. Vacuoles were detectable after 12 hours of incubation with the supernatant from *H. pylori* strains. The extent of vacuolation increased with the time of incubation, the monolayer of HEp-2 being completely vacuolated at the end of 48 hours. There was considerable variability in the cytotoxin activity produced *in vitro* by the *H. pylori* isolates. Out of the sixteen isolates ten isolates showed neutral red uptake equivalent to that of the reference strain 26695. Culture supernatants from KMC 03, KMC 04, MMC 03, MMC 04, KMC 156, KMC 157, KMC 159, KMC 182, KMC 190 and KMC 269 induced extensive cell vacuolation. The level of neutral red uptake in HEp-2 cells by vacuolation of these strains was comparable to that induced by the toxigenic reference strain. Four isolates MMC 01, MMC 02, KMC 166 AND KMC 292 showed less apparent neutral red uptake. These isolates did not induce any significant increase in neutral red uptake. Uninoculated broth medium did not
Hep-2 cells were incubated with culture supernatants from the standard and clinical isolates at indicated time points in the dilution of 1:1 and 1:2. The intracellular neutral red was quantified spectrophotometrically for the absorbance at 540 nm. Uninoculated broth was used as a control. KMC 01, MMC 02 and KMC 166 exhibited very less modulating activity. KMC 03, MMC 03, KMC 156, KMC 157, 159, 190 showed strong vacuolating activity. Other clinical isolates showed moderate vacuolating activity.
induce vacuolation on HEp-2 cells. Two isolates KMC 166 and KMC 292 lacked detectable vacuolating activity. The absorbance for the treated was subtracted with the background O.D i.e., uninoculated absorbance. The vacuolating activity of the culture supernatants was dose and time dependent. Maximum vacuolation was seen in 48 hours with 1:2 dilutions for the culture supernatant. At 24 hours the vacuolation of HEp-2 cells was visible with 1:1 dilution. This vacuolating activity could be compared to the VacA genotypes of these clinical isolates. Fig 3.2 shows the extent of vacuolation induced by culture supernatant of clinical isolates, quantitated by neutral red uptake assay and represented graphically. Uninoculated bacterial culture medium (Brain heart infusion broth) failed to induce cell vacuolation.

3.2 CYTOTOXICITY STUDIES OF THE OUTER MEMBRANE PROTEINS OF \textit{H. pylori} ON HEp-2 CELLS

Incorporation of radioactive thymidine is an index of the extent of a proliferation exhibited by a proliferating cell. Growing and dividing cells take up thymidine for incorporation in to DNA during active DNA synthesis. Reduction in the incorporation of thymidine in the presence of a bacterial protein indicates that the protein is cytotoxic or suppressive.

Toxic virulence factors have been reported from secretory, cytosolic and membrane fractions of \textit{H. pylori}. Initially the whole cell sonicate of the reference strain 26695 was analysed for its cytotoxic property. Dose response study of the whole cell sonicate of the standard strain for 24 hour showed cytotoxic effect at a concentration of 200 µg/ml (Fig. 3.3A). Lesser concentrations as 10 µg, 25 µg, 50 µg, of the sonicate did not show a significant difference in the thymidine uptake when compared to untreated
controls. The effect of various doses of the whole cell sonicate on HEp-2 cells at different treatment period is graphically represented in terms of cpm/mg protein in Fig.3.3A. The maximum inhibition of thymidine uptake was observed only with 200 µg/ml of the whole cell sonicate. The cytotoxic effect of the sonicate is almost similar to that of the live bacteria, thus creating a difficulty in identifying a new putative virulence-associated factor in a pool of proteins in the whole cell sonicate. Therefore cellular fractionation of the bacteria was carried out and their effects were analysed.

Outer membrane proteins in bacterial pathogens have a particular significance as a potential target for protective immunity and are therefore good candidates for the unknown proinflammatory virulence factor(s). OMP’S from four strains characterised by adherence and vacuolation assays were analysed for their strain variability with respect to cytotoxicity of OMP’S. These OMP’s were added to HEp-2 cells in various concentrations and the extent of cytotoxicity was analysed in terms of proliferation of HEp-2 cells by thymidine incorporation assay. Outer membrane proteins of all the strains caused concentration-dependent degeneration of the cells, although the titer of cytotoxic activity varied between strains. OMP’s from the clinical strains differing in their adherence and vacuolating property each induced significant inhibition of cell proliferation. In a dose response study, 10 µg, 25 µg, 50 µg and 100 µg of the OMP were treated with HEp-2 cells to analyse the minimum lethal dose that inhibits proliferation of HEp-2 cells. Lower doses such, as 10 µg and 25 µg did not show a drastic reduction in the thymidine uptake either at 2 hours or 4 hours. OMP at a concentration of 50 µg /ml showed a significant reduction in thymidine uptake by HEp-2 cells (Fig.3.3B). The minimum lethal dose of OMP mediated cytotoxicity was almost similar for all strains, except there was a marginal difference in the amount of
Fig. 3.3A Dose response study of the cytotoxic effect of the whole cell Sonicate of *H. pylori* on HEp-2 cells.

HEp-2 cells were treated with the whole cell sonicate from the standard strain 26695 in different doses as 10 μg, 50 μg, 100 μg and 200 μg at different time points. Whole cell sonicate at lower concentrations did not induce significant cytotoxic effect at earlier time periods. Only 200 μg of the whole cell sonicate at 24 hour was found to be cytotoxic. The controls were untreated HEp-2 control and TE treated control.
Fig. 3.3B Dose response study of the cytotoxic effect of OMP by Thymidine incorporation assay

HEp-2 cells were treated with different doses of the OMP from the standard and clinical isolates for 2 hours and 4 hours. The OMP of the clinical strains KMC 03 and MMC 03 showed similar cytotoxic levels to that of the ATCC 26695 strain. The dosage of cytotoxicity varied slightly for the clinical isolates KMC 01 and MMC 04.
thymidine uptake indicating differences in these strains in inhibition of proliferation. Time course analysis of the OMP mediated cytotoxic effect was done at various time points such as 30 min, 1 hour, 2 hour, 4 hour, 6 hour and 8 hour. Significant reduction in proliferation expressed as thymidine uptake was not observed at earlier time points till 2 hours. There was apparent reduction in thymidine uptake observed at a concentration of 50 µg at four hours and maximal between four and six hours (Fig.3.3C). The cells remained attached to the culture plates even after incubation for 3 hours beyond which the cells slowly become rounded up and begin to float. The clinical strains KMC 03 and MMC 03 showed maximum cytotoxicity at a concentration of 50 µg, beyond which the HEp-2 cells monolayer are detached from culture plates and become non-adherent at the end of 6 hours (Fig.3.4). The strains KMC 01 and MMC 04 exhibited less cytoxicity compared to the ATCC strain at the same concentration. Therefore the cytotoxic effect exerted by the OMP from adh+vac+ strains (KMC 03 & MMC 03) was greater than that of the OMP from the less virulent clinical isolates (KMC 01 & MMC 04). HEp-2 untreated and TE treated served as controls. Therefore the optimum concentration of OMP to achieve more than 50% cytotoxicity was 50 µg/ml.

3.3 DIFFERENCES IN THE CYTOPATHIC EFFECT EXERTED BY OUTER MEMBRANE PREPARATIONS OF H. PYLORI CLINICAL STRAINS

The cytopathic effects of any virulent factor/protein can be well studied with the change in the morphology of the host cell during in vitro studies. This change in the appearance of the host cells in response to the treatment with a virulent factor/protein is a preliminary, but a major event in understanding the pathogenic interaction between the bacteria and the host.
HEp-2 cells were treated with 50 μg of OMP of the standard strain and clinical strains at different time points. The OMP of the clinical strains KMC 03 and MMC 03 shows cytotoxic effect similar to that of the standard strain. The strains KMC 01 and MMC 04 exhibited less cytotoxicity at the indicated time point. Untreated HEp-2 and TE treated HEp-2 denotes the control. HEp-2 cell proliferation was monitored by [³H]-thymidine.

Fig. 3.3C Time course analysis of the cytotoxic effect of the OMP of *H. pylori*
The cytopathic effect leading to changes in the morphology of HEp-2 cells and their viability was analysed by trypan blue exclusion assay. Drastic changes in the spindle shape of HEp-2 cells were observed when treated with the OMP of *H. pylori*. The inner membrane and the cytosolic preparation from the standard strain and one highly virulent clinical strain MMC 03 did not show any significant morphological difference even at high protein concentration. The outer membrane preparation exerted severe cytopathic effect and there was difference in the morphological changes induced by different clinical isolates from less virulent to highly virulent. The viability of the HEp-2 cells and the structural changes induced by *H. pylori* OMP’s are shown in panel in Fig. 3.4. The HEp-2 cells become degenerated after treatment with the OMP’s from the standard and clinical isolates. The cytopathic effect induced by these OMP’s was dose and time-dependent phenomenon. HEp-2 cells treated with OMP’s from KMC 01 and MMC 04 caused elongation of cells (Fig. 3.4), but the cells did not get rounded up. The cell-cell adhesion was lost and the cells become shrunken with their spindle shape being intact. The cells remain attached to the culture plates even after four hours. HEp-2 cells treated with the OMP’s from KMC 03 and MMC 03 revealed severe lysis indicating a significant cytopathic effect or adverse pathological changes. At the end of four hours after OMP treatment the cells lose their spindle shape, become rounded up and subsequently become detached from the culture plates as floaters. Trypan blue exclusion assay to check the viability of the cells show the accumulation of trypan blue in the degenerated cells at 4 hours. Only the OMP’s from the strains KMC 03 and MMC 03 produced a similar degree of cytopathic effect as the standard strains. The increase in cytotoxic activity with time was observed for all the clinical strains.
The viability of the HEp-2 cells after exposure to OMP at indicated time points was checked by the trypan blue exclusion assay. At the end of 4 hours, the HEp-2 cells treated with OMP induced severe cytopathic effects resulting in rounding up of cells with standard strain and cell shrinking with KMC 01. HEp-2 cells exposed to the TE served as control. Whole cell sonicate and Inner membrane protein (IMP) at the same concentration did not induce significant cytopathic effect.

Fig. 3.4A. Morphological changes in HEp-2 cells due to cytopathic effect of OMP
Fig. 3.4B. Morphological changes in HEp-2 cells due to cytopathic effect of OMP

Cytopathic effect of OMP from the standard and clinical isolates of *H. pylori* on HEp-2 cell was checked by trypan blue exclusion assay. At the end of 4 hours, the HEp-2 cells treated with OMP from KMC 03 and MMC 03 caused degeneration of cells similar to the standard strain. OMP from MMC 04 induced shrinking of cells with loss of cell-to-cell adhesion. HEp-2 cells exposed to the TE served as control. Whole cell sonicate and Inner membrane protein (IMP) at the same concentration did not induce significant cytopathic effect.
3.4 USE OF OUTER MEMBRANE PROTEINS AS A CANDIDATE ANTIGEN IN IMMUNOBLOT ASSAY

Serological testing has recently been proposed as an aid in diagnosis of *H. pylori* infections. In this study, the immunoblot technique was used to evaluate the possibility of using different antigen preparations of *H. pylori* for this purpose. Various antigens checked by immunoblot analysis include, Whole cell sonicate, Acid-glycine extract and crude outer membrane protein preparations.

Colonised patients were defined as infected, who were culture positive, urease positive and positive for spiral bacteria by direct microscopic examination of biopsy specimen. Among these patient's, 7 non-ulcer dyspeptic patients, 12 duodenal ulcers, 2 gastric cancers, 4 gastric ulcer and 7 healthy controls were identified. Sera from seropositive patient’s tested by western blotting against whole cell sonicate revealed thirteen major bands and about 10 minor bands. However, all patients with *H. pylori* infection did not produce antibodies against a single polypeptide. Sera from control group i.e., the seronegative group revealed from 0 to 3 bands. Polypeptides with approximate molecular masses between 120, 90, 66, 50, 30, and 19 kDa seem to be the most specific polypeptides for the diagnosis of *H. pylori* infections. The minor bands varied from 10-12 and pattern of their appearance also varied. This study showed only minor differences in antigenic composition between different clinical isolates of *H. pylori*. A set of blots with sera from seropositive patients is depicted in Fig.3.5A (Panel A & B). The frequencies of these immunoreactive bands were compared with the disease status, which failed to demonstrate any significant relationship between the different disease conditions and antibody pattern.
The acid glycine treatment of *H. pylori* cells is a mild method for releasing surface proteins. The protein profile of the acid glycine extract was checked by 10% SDS-PAGE. Ten patient's sera were analysed in an immunoblot assay with glycine-extracted antigens separated by SDS-PAGE. Immunoblot patterns obtained with sera from the different groups are presented in Fig.3.5A (Panel D). The immunoblot with acid glycine extract of *Helicobacter pylori* proteins showed reproducible immunoreactive bands at molecular weights of 130, 93, 67, 55, 43, 30, and 20 kDa. The immunoreactive bands between 66 and 30 kDa were not present in all sera and was found to be of minor immunological significance. No correlation between specific immunoblot patterns and clinical signs induced by *Helicobacter pylori* infection was observed. In the present study, an immunoblot assay with a crude *H. pylori* outer membrane protein preparation (HPOmp) as an antigen was designed. The crude outer membrane preparation prepared by Sarkosyl insoluble method was analysed for its protein profile by SDS-PAGE (10%). The main proteins seen in membrane preparation appeared to be large and small urease subunit based on their molecular sizes. There were no major differences between the OMP protein profiles between the clinical strains. The immunoreactivity of the crude OMP against serum samples obtained from *H. pylori* infected patients was checked by immunoblot analysis. Immunoblot analysis of sera from seropositive patients revealed 12 major bands. Of these bands the proteins with molecular masses of approximately 130, 97, 66, 50, 43 and 29 kDa was the major immunoreactive band appearing irrespective of the disease condition (Fig.3.5B). The other minor bands between 66 and 43 kDa were of minor immunological significance with the frequency of reactivity being less. The lower molecular mass proteins corresponding to porin proteins also demonstrated significant immunoreactivity. The high molecular protein 130 kDa corresponding to cagA and 97 kDa corresponding to vacA showed significant...
Fig. 3.5A Immunoblot patterns of the patients sera against the whole cell sonicate and the Acid-Glycine extract

A set of sera from *H. pylori* infected subjects were analysed for their immunoreactive pattern against two antigenic preparations: whole cell sonicate and Acid-Glycine extract. The pattern of immunoreactive bands obtained with the whole cell sonicate of the standard strain 26695 with a set of patients sera infected with *H. pylori* are shown in panel A and panel B. Panel C represents the immunoblot with the sera from the healthy controls. Immunoblot pattern in Panel D shows the antibodies to Acid-Glycine extract in the patients’ sera. Lane 11 and 12 in panel D is the control group, which did not reveal any immunoreactive bands. Molecular masses are indicated on the left.
Fig. 3.5B  Immunoreactivity of the patients’ sera against Outer membrane proteins of *Helicobacter pylori*

Antibodies to the OMP of 26695 (standard strain) obtained with a set of patients’ sera. Reproducible immunoblot pattern was observed with different disease conditions. The major immunoreactive bands appeared at 66, 50, 43 and 29 kDa (Panel A & B). Negative controls from healthy subjects are shown in panel C. Molecular masses are indicated on the left.
immunopositive reaction. Fig. 3.5B shows the immunoblot pattern of patient’s sera against crude OMP antigen preparation. Negative sera was represented by sera from healthy controls, children, histectomy cases etc.,

3.5 CYTOKINE RESPONSE OF EPITHELIAL CELL LINE TO H. PYLORI STIMULATION in vitro

3.5.1 Role of OMP in cytokine induction

Cytokines play a major role in mobilising cellular defence mechanisms to eliminate pathogens. The expression of cytokines may be the initial signal for the acute inflammatory response following bacterial invasion of mucosal surfaces. In vivo gastric infection with H. pylori induces the mucosal production of various cytokines in the host thus playing a role in the immunopathogenesis of H. pylori infection. Specific cytokines have also been shown to enhance the development of cancers. Therefore a modified in vitro system was applied to investigate whether direct bacterial adhesion to epithelial cells and/or bacterial products which are able to pass the epithelial barrier trigger cytokine release. In the present study we showed the cytokine expression induced by live H. pylori and OMP’s using the RT-PCR technique. It was also investigated whether there are substantial differences among wild-type H. pylori and clinical samples.

A RT-PCR assay with the primer pairs specific for IL-6 and IL-8 cytokines was employed. The capacity of HEp-2 cell line to produce IL-6 and IL-8 upon coculture with live H. pylori was first examined. HEp-2 cells were exposed to the live H. pylori and the crude OMP preparation from the standard clinical isolates for a period of time and the total mRNA extraction was done followed by Reverse transcriptase PCR with the primers for cytokines IL-6 and
IL-8. Coculture with the live bacteria for 4 hours enhanced significantly IL-6 and IL-8 production by HEp-2 cells. The time for the analysis of IL-6 and IL-8 mRNA transcripts was similar to that of the adherence assay. We examined the capacity of the clinical isolates to induce IL-6 and IL-8 production. All isolates were positive for vacA irrespective of their ability to induce cytokine production. These isolates were able to induce cytokine production in spite of the varying difference in the adherence and cytotoxic effect. LPS was included as a positive control. Amplification of the same cDNA with the primers for β-actin as a housekeeping gene demonstrated that the expression of transcript for this constitutive protein was unaffected. Fig. 3.6 A shows the increase in IL-8 and IL-6 specific message production following stimulation with live *H. pylori* cells relative to the constant level of β-actin message.

With the outer membrane protein showing cytotoxic effect, the ability of OMP to induce chemokine secretion was analysed. The HEp-2 cells were treated with the OMP's of the standard strain and the characterised clinical isolates by in vitro cytokine stimulation assay. Using the conditions specified above, we studied the kinetics of OMP induced cytokine mRNA expression in HEp-2 cells. As detected by RT-PCR, both IL-6 and IL-8 mRNA transcription was evident in HEp-2 cells within 30' of exposure to the OMP. Induction was maximal after 1 hour and no expression was observed with treatment of OMP at later hours of incubation. Fig. 3.6 B shows the kinetics of IL-6 and IL-8 mRNA transcripts after 30' and 1 hour exposure of HEp-2 cells to OMP. All *H. pylori* clinical isolates were phenotypically similar except for the recognised differences in the expression of vacA in terms of vacuolating activity and allelic type. All strains showed significantly increased levels of IL-6 and IL-8 with no major difference. LPS treated HEp-2 cells were taken as a positive control. β-actin control was used as a house keeping gene control.
Fig. 3.6A RT-PCR analysis of mRNA using primers specific for cytokines IL-6 and IL-8

HEp-2 cells exposed to live bacteria for 4 hours and the mRNA was used for RT-PCR analysis with primers for IL-6 and IL-8. Live bacteria induce IL-6 and IL-8 mRNA. There were no differences observed in the levels of cytokine expression induced by different clinical strains. LPS treated HEp-2 was used as a positive control. Untreated HEp-2 was used as a negative control. β-actin was used as a housekeeping gene control.
HEp-2 cells were treated with OMP for indicated points of time. Following mRNA extraction, RT-PCR shows the amplification product for IL-6 and IL-8 with specific primers. Panel A indicates the HEp-2 cells treated with OMP for 30 minutes. Panel-2 indicates the HEp-2 cells treated with OMP for 1 hour. Lane 1 represents the untreated HEp-2 control. Lane 2 shows the expression induced by OMP from the standard strain 26695. Lane3-6 indicates the cytokine expression induced by the OMP from the clinical isolates. Lane 7 is the LPS treated positive control. β-actin was used as house-keeping gene control.
NF-κB, a ubiquitous nuclear transcription factor plays an integral role in regulating the human immune response. NF-κB is activated upon stimulation by a large variety of pathogenic agents. It has been already reported that exposure of gastric epithelial cell lines to *H. pylori* potentially activates NF-κB. Also activation of NF-κB is critical for the inducible expression of proinflammatory response genes including cytokine.

In order to investigate whether crude outer membrane protein preparation is required for NF-κB activation, HEp-2 cells were treated with various OMP’s from a standard strain as well as the clinical isolates. Subsequently, the cells were harvested and total cell extracts were prepared and assayed for NF-κB DNA binding by an electrophoretic mobility shift assay (EMSA). Previous reports show that maximal DNA-binding of NF-κB was observed between 90 and 180 min post infection and was not detectable later than 240 min post infection. In this study subconfluent layers of HEp-2 cells were treated with OMP from the standard and clinical isolates. At different time points, post challenge nuclear protein extracts from HEp-2 cells were prepared and analysed for the levels of NF-κB activation. An enhanced binding of NF-κB was observed within 30’ post treatment and was seen until an hour (Fig. 3.7A). To determine whether NF-κB activation is sustained at later stages the incubation of HEp-2 cells after 1 hour with the OMP was checked. Maximal DNA-binding of NF-κB was observed between 30’ and 1 hour of treatment and was not detectable later than 1 hour of treatment (Fig. 3.7B). The OMP from standard strain 26695 showed maximal activation compared to the clinical isolates (Fig. 3.7B). There was no marked difference in the activation of NF-κB between
Fig. 3.7A  Activation of NF-κB induced by the *H. pylori* OMPs

The effect of OMPs from various *H. pylori* isolates on NF-κB binding in HEp-2 cell line. EMSA of HEp-2 cells treated with 50μg of OMP for 30 min (A) and 1 hour (B). Untreated HEp-2 cells were taken as negative control. TNFα treated HEp-2 cells is the positive control. The arrow indicates specific NF-κB complexes. The closed circle indicates non-specific binding to the probe and the square denotes unbound oligonucleotide.
Fig. 3.7B Activation of NF-κB induced by the *H. pylori* OMPs

EMSA of HEp-2 cells treated with 50 μg of OMP show NF-κB activation at 30 min and 1 hour (fig. 3.7A). Maximal NF-κB–DNA binding was seen at 1 hour and no activation was observed at 2 hour and 3 hour timepoint. Untreated HEp-2 cells was taken as a control. TNFα treated served as positive control. The arrow indicates specific NF-κB complexes. The closed circle indicates nonspecific binding to the probe and the square denotes unbound oligonucleotide.
clinical isolates. The DNA binding activity was induced to similar extents by the OMP preparation from the clinical isolates. Fig. 3.7A & B shows the effect of OMP from various clinical isolates on NF-κB DNA binding in HEp-2 cells.

3.7 CYTOSKELETAL REARRANGEMENTS INDUCED BY HELICOBACTER PYLORI STRAINS AND ITS OMP IN VITRO

3.7.1 Actin depolymerisation in HEp-2 cells treated with the OMPs of Helicobacter pylori

Helicobacter pylori colonisation of the gastric mucosa induces peptic ulcer disease and interferes with ulcer healing. Re-epithelialisation is an essential component of ulcer healing requiring cell migration and proliferation, which are dependent on the cell cytoskeleton. In vitro adherence of H. pylori to gastric epithelial cells is associated with reduced cell proliferation contributing to ulcer formation and delayed ulcer healing. Cell proliferation and migration, both essential for mucosal healing are dependent on the cell cytoskeleton.

The relationship between Helicobacter pylori adherence, cytotoxic effect of OMP’s, and modification of the cytoskeletal structure was investigated by studying the effects of four H. pylori strains cocultured with HEp-2 epithelial cells and compared with the standard strain. These bacterial strains were isolated from patients with peptic ulcer disease or nonulcer dyspepsia and categorised according to their extent of adherence and degree of vacuolation. HEp-2 cells were infected with H. pylori at a concentration of 2 x 10⁸ cells and the host cell cytoskeletal rearrangements associated with H. pylori attachment was observed using by fluorescence microscopy. The actin cytoskeleton of HEp-2 cells stained with fluorescein-conjugated phalloidin expressed actin filaments in the form of short stress fibers and thin network at the edges when
treated with the live bacteria. Fluorescein-conjugated phalloidin staining of HEp-2 cells showed that \textit{H. pylori} adherence stimulated expected reorganisation of host cell actin. The actin filaments were found to accumulate below the adherent \textit{H. pylori}.

The ability of the outer membrane proteins to mediate adherence to the gastric mucosa has not yet been elucidated. By thymidine incorporation assay the cytotoxic role of the OMP is clear. Therefore, OMP mediated host cell cytoskeletal changes was further analysed by Fluorescent actin assay (FAS). As per the dose response study, a dosage of 50 \( \mu \)g of OMP from the standard and different clinical isolates was used for the FAS assay. HEp-2 cells were treated with the OMP for 4 hours and the host cell actin was stained with fluorescein-conjugated phalloidin. Distinct fluorescence was seen all around the periphery of cells indicating the accumulation of depolymerised actin (Fig. 3.8A). There was not much significant difference in the actin polymerisation and accumulation induced by OMP from the different clinical isolates. The extent of actin depolymerisation and punctuated spots depicting the focal adhesion occurred more when treated with the strains with more vacuolating activity. An intense F-actin fluorescence was not observed in the case of OMP treatment. An intense F-actin condensation and OMP mediated actin depolymerisation and actin accumulation was not observed significantly in \textit{H. pylori} infection.

3.7.2 Disruption of tubulin network in OMP treated HEp-2 cells

Microtubules are polymers that form a network framing a cytoskeleton in the cells. In this study the changes in the tubulin network of cytoskeleton in HEp-2 cells following \textit{H. pylori} infection was analysed. HEp-2 cells were
Fluorescent Actin Staining

Fluorescein-conjugated phalloidin staining of HEp-2 cells treated with OMPs from the reference strain and the clinical isolates. The untreated HEp-2 cells were taken as control show a loosely organized network of actin filaments with densely fluorescent cell borders. HEp-2 cells treated with OMPs demonstrate small foci of intense fluorescence indicating the actin filament polymerization as shown by fluorescence microscopy.
HEp-2 cells when exposed to *H. pylori* exhibit rearrangements in the tubulin network. The cells show peripheral accumulation of tubulin forming a ring (26695, KMC 03, and MMC 03) or the accumulation of tubulin in the centre of HEp-2 cells as in the case of KMC 01 and MMC 04. Untreated HEp-2 cells were used as a control.
Fig. 3.8C Cytoskeletal rearrangement of tubulin induced by OMPs of *H. Pylori*

HEp-2 cells were treated with OMPs from the standard 26695 and clinical isolate. The tubulin cytoskeletal structure become disrupted upon exposure to OMP and accumulation of tubulin in the centre of HEp-2 cells and peripheral distribution of tubulin is observed. HEp-2 cells untreated is used as a control.
infected with the live bacteria for 6 hours and the changes in the tubulin pattern were observed. When ATCC was infected with HEP-2 cells the cells become vacuolated with the tubulin network forming a peripheral ring inside the cells. The strongly adhering strains and with more vacuolating activity formed a peripheral accumulation of tubulin network (Fig 3.8B) while the other 2 clinical isolates induced a condensation of tubulin at the center of the cells. The cytostatic effect of the OMP inhibiting proliferation of the host cells was observed with the cell static at particular events of cell cycle. After the HEP-2 cells were treated with the OMP from various clinical strains there was significant detectable effects on the microtubule network or cell growth (Fig 3.8C). The spatial organisation of the tubulin cytoskeletal network becomes disorganised microtubule pattern with subsequent peripheral accumulation and condensation at the center of the cells forming microtubule centers.

3.8  H. PYLORI OUTER MEMBRANE PROTEINS INDUCES EPITHELIAL CELL APOPTOSIS IN A CELL FREE SYSTEM

Microbial pathogens or their products can directly activate the cell death-signaling cascade. Infection with  H. pylori  activates a proinflammatory gene program in human gastric epithelial cells and is associated with significant epithelial cell damage, including increased levels of mucosal apoptosis. This enhanced gastric epithelial cell apoptosis observed during infection with Helicobacter pylori has been suggested to be of significance in the etiology of gastritis, peptic ulcers, and neoplasia. Recently several factors have been implicated in the induction of apoptosis. The outer membrane proteins are potential candidates as virulence factors and thus its role in apoptosis was investigated.
**Fig. 3.9A** Apoptosis in *H. pylori* infected HEp-2 monolayer.

Apoptotic cells in *H. pylori* infected cells with the standard strain and clinical isolates after 18 hours by propidium iodide staining in fluorescence microscopy. HEp-2 untreated control show normal morphology. *H. pylori* infected demonstrate morphologic features of apoptosis including condensed chromatin with apoptotic body formation.
Fig. 3.9B Induction of apoptosis by H. pylori OMPs
HEp-2 cells when treated with OMP from the standard and clinical isolates demonstrate apoptotic morphology as shown by fluorescence microscopy stained with propidium iodide. HEp-2 cells untreated is used as a control. Apoptotic cells show condensed chromatin with apoptotic body formation.
3.9 CHARACTERISATION OF vacA AND cagA ALLELES OF THE INDIAN CLINICAL ISOLATES

VacA and cagA are specific virulence determinants on *H. pylori* strains that influence the clinical outcome of infection. Specific vacA genotypes are identified which are characterised by the differences in the signal sequence and the middle region of the gene and also only 50-65% of the strains produce the active cytotoxin. The cagA gene is present in about 60-70% of *H. pylori* strains encoding a molecular weight protein. The gene encoding the cytotoxin, vacA has a mosaic structure consisting of regions that are relatively well conserved among all strains and regions that are conserved only among a proportion of strains. The two best characterised of the latter regions are the signal sequence, which has three allelic types (s1a, s1b, and s1c), and the mid-region, which has two allelic types (m1 and m2) (Atherton et al., 1995). Both signal sequence and mid-region types are independent predictors of the *in vitro* cytotoxin activity of a strain. Also cagA gene is a second non-conserved characteristic gene of *H. pylori* shown to be a marker for increased ulcer risk (Crabtree et al., 1991). In this study the characterisation of vacA alleles and analyses of the differences in the cagA gene that are present in the Indian clinical isolates was undertaken.

Twenty-four strains were isolated from *H. pylori*-positive patients with dyspeptic symptoms. Endoscopic examination revealed duodenal ulcers in 13 patients, Non-ulcer dyspepsia in 5 patients, gastric ulcers in 4 patients, and gastric cancer in 2 patients. These strains were confirmed as *H. pylori* by culture, gram stain morphology, Urease, catalase, and oxidase tests. Genomic DNA was extracted by CTAB/NaCl method and used for PCR amplification with the specific primers.
3.9.1 Determination of vacA genotypes and cag status and relationship to gastrointestinal Diseases

The vacA gene was detectable in all H. pylori isolates. The primers vac1F and vac1R was used to amplify the vacA signal sequences and a PCR product of either 201 or 228 bp is predicted. PCR products of either 201 or 228 bp were obtained from all the 24 isolates. Seventeen of 24 strains yielded the smaller product, representing the genotype s1, and 7 yielded the larger product, representing genotype s2. A PCR product of any other size was not detectable with any of these clinical strains (Fig. 3.10).

All these isolates were then checked for the differences in the middle region of the vacA gene. These clinical isolates and a reference strain was then amplified either by the primers vac3F and 3R representing the type m1, or the primers vac4F and 4R specific for type m2. Nineteen isolates were classified as type m1 yielding a product size of 388 bp (Fig. 3.11) and 5 isolates were classified as type m2 with a product size of 342 bp (Fig. 3.12) for the primers vac4F and vac4R. Among these eight isolates had DNA amplified by both primer sets. Among these 24 isolates studied all the four possible combinations of signal sequence and middle region were identified. The s1-m1 combination was observed in 14 isolates, and s1-m2 combination was seen in 5 isolates. The s2-m2 and s2-m1 combination was identified in 2 isolates and 3 isolates respectively. Infection with a type s1 strain was found in 8 duodenal ulcer patients, all the 5 nonulcer dyspepsia patients and 2 gastric ulcer patients and in 1 gastric cancer patient. Six of the duodenal ulcer patients and 1 gastric cancer patient were of the type s2 strain. The s1-m1 and s2-m2 combination was not associated with the disease status.
Fig. 3.10 Agarose gel electrophoresis after PCR amplification of H. pylori DNA using primers for the signal sequence.

Genomic DNA from the clinical isolates of H. pylori was checked for vacA allelic typing by PCR with the specific primers for signal sequence of the vacA gene. 17 out of the 24 isolates were of the s1 type with a product size of 201bp. Seven isolates were s2 type with a product size of 228bp. Lane M indicates the 100bp ladder. Lane 1 is the positive control (26695). Lane 14 & 27 is the primer control.
Fig. 3.11  PCR products differentiating the \textit{vacA} allele as type m1 in clinical isolates of \textit{H. pylori}

Agarose gel electrophoresis after PCR amplification of \textit{H. pylori} isolates using primers for the middle region for \textit{vacA} gene. Lanes: M- 100 bp DNA ladder. Lanes 4, 6, 7, 21 and 23 did not yield any product with the primer Vac3F-3R representing the m2 genotype. Out of 24 isolates, 5 isolates are of the type m2. 19 isolates yielded the PCR product of the size 388bp with the primer vac3F-3R representing the type m1.
Fig. 3.12 PCR products differentiating clinical isolates of *H. pylori* as type m2

Agarose gel electrophoresis after amplification of the PCR product with specific primers vac4F-vac4R for the middle region of the *vacA* gene. The type m2 yields the product size of 342 bp. Out of 24 isolates, 5 were only m2. 8 isolates yielded both the product sizes of 388bp and 342 bp for the primers vac3F-3R and vac4F-4R respectively. Lanes: M-100 bp DNA ladder. Lanes 1 &15 are primer control. Five isolates in lane 4, 6, 7, 21 and 23 was amplified only by vac4F-4R representing type m2.
3.9.2  CagA gene status in the Indian clinical isolates and size variation

Twenty of the 24 strains were cagA+ strains. Among these strains PCR products were obtained with both the cagA primer sets for the region in the cagA gene. Nine of 13 strains isolated from duodenal ulcer carried the cagA gene. All the two isolates from patients with gastric cancer were cagA+. The two primers sets designed were aimed to check the hydrophilic region and the region of internal duplications of the cagA gene, which may be responsible for the cagA size heterogeneity. The PCR products of cagA+ strains obtained with primer designed for the hydrophilic region yielded a product size between 612-615 bp. Fig. 3.13 show PCR amplification of H. pylori isolates with primers cag1-cag3. The PCR products amplified in the region of internal duplication differed in size varying from 450 bp in 4 isolates, to between 500 and 600 in 8 isolates and above 600 bp in 8 isolates. Fig. 3.14 shows the heterogeneity in the PCR products after amplification of H. pylori DNA with the primers cag-cag4.
Fig. 3.13 Analysis of the prevalence of the \textit{cagA} gene in the clinical isolates of \textit{H. pylori} by PCR amplification of the 3' hydrophilic region of the \textit{cagA} gene

Agarose gel electrophoresis of the PCR products after amplification of gDNA with the specific primers for the hydrophilic region of the \textit{cagA} gene. Out of 24 isolates, 20 isolates were \textit{cagA} positive yielding a product size between 612-615bp. Lane M indicates the 100bp ladder, Lanes 1 & 15 Positive control of the standard strain 26695. Four isolates (lane 4, 7, 16 and 23 did not yield any product indicating a \textit{cag}^- genotype.
Fig. 3.14 Prevalence and size variation of the \textit{cagA} gene in the clinical isolates of \textit{H. pylori} by PCR amplification of the region of internal duplication of the \textit{cagA} gene.

Agarose gel electrophoresis of the PCR product after amplification with the specific primers cag2-cag4 for the region of internal duplication in the \textit{cagA} gene. The PCR products amplified with the clinical isolates for the region of internal duplication of the \textit{cagA} gene varied in size between 450bp to 700bp. Lane M-100bp ladder. Lane 1-Positive control, Lane 26-Primer control.