ORIGINAL ARTICLE

EFFECT OF H. PYLORI PROTEINS JHP 0290 (FL) AND JHP 0290 ON HOST CELL BEHAVIOR

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ABSTRACT

Objective: This study was designed to investigate the effect of H. Pylori proteins JHP 0290 (FL) and JHP 0290 on host cell response.

Materials and Methods: This study was conducted at Khyber Teaching Hospital (KTH), Peshawar from April 2021 to September 2021. H. Pylori genomic DNA (J99 and 26695), pET expression system plasmid (PET21a, 22b & 28b+), kanamycin 50μg/ml and ampicillin 100μg/ml, LB agar plates and LB media, Talon@IMAC resin column were used for studying H. pylori proteins. Genes coding for these proteins were successively cloned into PET21a, PET22b, and PET28b+ expression vectors but no expression was seen with different concentrations of IPTG in BL21(DE3) culture, and expression was prolonged for 4hrs.

Results: Only JHP0290 (FL) & JHP0290 (-17a.a) were successively cloned in PET28b+ and expression was induced by using 100μM IPTG in E.coli strain (BL21DE3). Restriction analysis showed the size of JHP0290 (FL & -17a.a) at approximately 550bp and protein size was analyzed on SDS-page which was 20Kda approximately. JHP0290FL protein was found in pellets while JHP0290 (-17a.a) protein was found in pellets & supernatant before being applied to Talon cell through resin column to get purified protein. The study showed a clear band of JHP0290 (FL) on SDS-page with imidazole eluted fraction pooled, while a faint band with a distinct band of JHP0290 (-17a.a) was also seen on SDS-page.

Conclusion: These changes are indicative of altered host cell response induced by H.pylori protein JHP0290 suggesting its possible involvement in the development and severity of the disease.

Keywords: H.pylori proteins, virulence factor, host cell response, genomic DNA, pathogenicity.

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INTRODUCTION

Various gastric diseases such as chronic gastritis, peptic ulcer, gastric cancer, and mucosa-associated lymphoid tissue lymphoma are caused by H. pylori.[1-3] H. pylori is present in about 70% of the world's population[4]. Because of its heterogeneous nature, H. pylori is perfectly adapted for survival in the gastric environment, and for the same reason, it is not so easy to detect the major bacterial factors that are directly associated with etiopathogenesis[5,6]. Virulence factors such as genes within the cag (cytotoxin-associated antigen) pathogenicity island encoding for CagA protein, polymorphic variation in the VacA vacuolating exotoxin, and the blood group antigen binding adhesions (BabA, SabA) are all possible bacterial factors[7,8]. In addition, duodenal ulcer-promoting gene (dupA), bacterial factors such as peptidoglycan, lipopolysaccharide (LPS), g-glutamyl trans-peptidase (GGT), and protease HtrA are all possible causes of pathogenicity[9,10,11]. The presence of these virulence factors is variable among H pylori found in different geographic areas and ethnic groups thus explaining the differences in disease prevalence among individuals e.g., Inhabitants of East Asia have the highest rate of gastric cancer in the world where almost all H. pylori isolates are cagA genopositive, vacA s1/i1/m1 and BabA expressing[12]. In order to understand the pathogenic mechanism of H.pylori, it is very important to identify and

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determine the functions of all proteins present in their cell. The exact and complete identification of proteins secreted by H. pylori is difficult because of its high lysis frequency that shows the outcome of the nonspecific release of all cytoplasmic proteins[13]. One protein which has been reported in the external medium by different researchers is HP1286. The prime sequence shows that HP1286 tends to belong to Y-Cell like a family of protein [14]. Structurally the Y-Cell like family is a subgroup of the lipocalin superfamily. Lipocalin has a prototype which is a retinol-binding protein (RBP) present in the plasma of higher animals [15].

Gastric inflammation and epithelial damage contribute to the secretion of proteins by H. pylori. Urease subunit beta UreB is a known cytoplasmic protein that has 0.25 ratios in the supernatant as that of the pallet and later on, it was considered a lysis index.

The ratio greater than UreB was used to differentiate specific proteins selectively released into the medium. About sixteen different proteins were found in the supernatant. These proteins may be implicated by H. Pylori-induced effects on gastric epithelium [16]. The aim of the study was to investigate how H. pylori proteins JHP 0290 (FL) and JHP 0290 affect the host cell response.

MATERIALS AND METHODS

This study was conducted at Khyber Teaching Hospital (KTH), Peshawar from April 2021 to September 2021. Two strains of H. Pylori genomic DNA (J99 and 26695), pET expression system plasmid (PET21a, 22b & 28b+), kanamycin 50μg/ml, and ampicillin 100μg/ml, Lysogeny Broth (LB) agar plates and LB media, Talon@ IMAC resin column were used.

AMPLIFICATION OF GENES USING PCR

H. pylori genomic DNA from the strain of J99 and the gene coding for JHP0290 (Full length & 18-184a.a) was amplified by PCR using high fidelity DNA polymerase from Finnzymes®. The forward and reverse primers contained NdeI and XhoI restriction enzyme recognition sequences sites. Isolation of circular and recombinant plasmid from DH5α

A single colony from a selective plate of plasmid in DH5α was taken and inoculated into 10ml LB media containing antibiotics (50μg/ml kanamycin or 100μg/ml ampicillin depending upon selection marker) followed by overnight incubation at 37°C with a shaking speed of 150 rpm. The vector was isolated from overnight culture by using the Omega-biotek plasmid miniprep Kit-I following the manufacturer's instructions [17].

Restrictive Digestion of Genes with XhoI and NdeI The restriction enzymes FastDigest XhoI and NdeI were used to digest the insert. The digestion was performed according to the protocol of Fermentas®. The digestion mix was then incubated at 37°C for 2 hours and analyzed on 1% agarose gel with undigested & 1Kb marker. Restrictive Digestion of Expression vector (pET system) with XhoI & NdeI

The expression vector (pET system) was also digested with the same restriction enzymes as with the insert to facilitate ligation. Purified 1.5μg of plasmid DNA was used to digest with XhoI & NdeI. After incubation for 1 hr, the total volume of the digested vector was loaded on the 1% agarose gel. The PCR products and digested plasmids from the gels were then purified by Gelifesciences PCR purification kit. Finally, the concentration of DNA (PCR products & pET plasmids) was quantitatively measured at 260 nm & 280 nm with nuclease-free water as a reference. Purification of PCR Products and plasmid (Quantification of DNA)

The purified and digested PCR products were cloned into pET expression vectors. The molar ratio (5:1) was used to ligate insert into the vector. Fermentas protocol for ligation was used to ligate the gene into the expression vector. The ligation mix was then incubated at 16^{0}C overnight and one without ligase enzyme as a negative control to test self-ligation. After overnight incubation, a ligation mix was used to transform DH5α competent cells.

Preparation of E.coli [DH5α + BL21 (DE3)] competent cells E.coli strains [DH5α + BL21 (DE3)] were supplied by Novagen and followed the method proposed earlier [18].

Transformation in DH5α competent cells The ligation mix 10μl was inoculated into 100μl DH5α competent cells and kept on ice for 30 minutes, followed by a heat shock at 42^{0}C for 1 min. The transformation was carried out as described earlier [19].

PCR analysis of clones 20 blocks were drawn on an LB agar plate containing kanamycin (50μg/ml) and a single colony was taken from a ligation mix plate and streaked on the blocks followed by inoculating the same loop containing the inoculum into a sterile Eppendorf tube having 20μl nuclease-free water. After that, Eppendorf tubes were incubated at 95^{0}C for 10 minutes, followed by centrifugation at 13000 rpm for 2 minutes. 2μl from supernatant from each Eppendorf tube was taken and used as template DNA in PCR reaction mix for verification of successful ligation and clone [20]. 12 colonies were taken from the ligation mix and one from positive control (circular Jhp0290 FL in PET28b+). After PCR cycle completion, PCR samples were loaded with 6x loading dye on 1% agarose gel for the verification of clones.

Recombinant plasmid isolation, gene amplification, and restriction analysis of clones 2,4,6,8 (Jhp0290-17a.a) no. clones were selected for plasmid isolation and amplification of gene of insert (Jhp0290-17a.a). PET28b+ (Jhp020 (-17a.a) clones (2, 4, 6 & 8) were inoculated into 10 ml LB media containing 50μg/ml Kanamycin (10μl) followed by incubation inside at 37°C incubator with shaking for overnight. Using Omega-biotek plasmid miniprep Kit-I, the plasmid was isolated following manufactures specification to analyze the band of interest [21].Transformation of Recombinant Plasmid in BL21 (DE3) competent cells

2, 4 (Jhp0290-17a.a in PET28b+) clones were selected for transforming in BL21 (DE3) competent cells and induced expression upon the addition of IPTG. The purified recombinant circular plasmid 1μl (80ng of DNA) was inoculated into 100μl BL21(DE3) competent cells and kept on ice for 30mins followed by a heat shock at 42^{0}C for 1 min. Then 400μl of pre-warmed LB media was added to the transformation mix and kept the mix at 37^{o}C with a shaking speed of 150 rpm/ hour. After 1 hour, 250μl reaction mix was placed on LB agar plates containing kanamycin (50μg/ml), and glycerol stock of JHP0290 FL in PET28b+ was also streaked on LB agar plate containing Kanamycin followed by incubation for overnight. After overnight incubation, colonies appeared 148 on 2 no. clone plates and 158 on 4.

Small scale (rapid) and large scale expression of Jhp0290 (-17a.a) and Jhp0290 (FL) These were carried out as described earlier for analysis of protein induction[21].

Purification of recombinant protein Jhp0290 (-17a.a) and Jhp0290 (FL) Jhp0290 (-17a.a) & FL proteins were purified by using the Clontech Talon manual and followed the instruction of usage [22]

Purification of protein Jhp0290 (-17a.a) and Jhp0290 (FL) TALON cell through resin TALON is an immobilized metal affinity chromatography (IMAC) resin charged with cobalt, which binds to his-tagged proteins with higher specificity than nickel-charged resins. As a result, TALON resin delivers his-tagged proteins of the highest purity [23]. Data were analyzed using Graph Pad Prism version 9.2.0 and Graph Pad InStat version 3.10.

RESULTS

After completion of PCR cycles, the samples were loaded on 1% agarose gel with a 1kb marker. PCR products were successfully amplified by Phusion high-fidelity DNA polymerase. PCR product was around 550bp in size which was compared to 1Kb 500bp marker band size as a reference. The amplified PCR products were further purified from the gel by Omega bio-tek and digested with XhoI & NdeI restriction enzymes at 37^{o}C. The digested vector was loaded on 1% agarose gel and digestion was carried out for further analysis. For the ligation of the construct into the PET expression vector, 80-100ng of plasmid and 35-45ng of PCR product were used. The construct was successively ligated into PET28b+. Colonies appeared on positive control (full lawn); ligation mix and no colonies were obtained on self-ligated mix plates.

PCR analysis of Clones12 colonies were taken from a ligation mix plate & one with positive control and used for PCR analysis of clones. PCR analysis was successful and bands of insert (Jhp0290-17a.a & FL) were seen on the gel. The size of colony bands was 550bp compared to the band of 500bp on the 1Kb ladder (Figure 1).

Plasmid amplification and purification The plasmid-containing insert was amplified and purified using the Omega-biotek plasmid miniprep Kit-I spin protocol. Quantification of the plasmid DNA was yielded in Table 1.

Amplification of insert from recombinant plasmid 2, 4, 6 & 8 clones (Jhp0290-17a.a in PET28b+ & positive FL) were selected for PCR to verify the correct size of gene of interest and PCR reaction mix protocol was the same as that for Colony PCR. PCR reaction yielded 550bp fragment on 1% agarose gel. On the 6th lane positive control was loaded and similar band can be seen with size range of 550bp (Figure 2).

Restriction analysis of Clones Recombinant plasmid was digested with two restriction enzymes XhoI & NdeI and loaded on 1% agarose gel that yielded 550bp and 5.4kb fragments in first four lanes after 1Kb ladder marker. On 6th lane a positive control (Jhp0290FL in PET28b+) also yielded 550bp fragment and no fragment occurred in negative control (Figure 3).

Small scale expression of Jhp0290-17a.a & Jhp0290 FL and SDS-page Two clones (Jhp0290-17a.a in PET28b+) were induced under the control of 100μM & 500μM IPTG at 2.5hrs incubation at 37^{o}C and one without IPTG with 0 hour & 2.5 hrs incubation times. The insert (Jhp0290-17a.a) was induced at 100μM & 500μM IPTG concentration and compared with uninduced at 0 & 2.5hrs time incubation. The size of the expression of gene is 20Kda approx. There is no expression of gene in un-induced at 37^{o}C at 0 hr & 2.5hrs (Figure 4).

Figure 5 shows the 2, 4 clones (Jhp0290-17a.a in PET28b+) induced by IPTG with 100μM & 500μM concentration and one clone (Jhp0290 FL in PET28b+) with 100μM IPTG concentration. The gene of interest was expressed at 100μM & 500μM but no expression seen in uninduced sample at 0hr & 2.5hrs at 37^{o}C. Large scale expression of Jhp0290-17a.a & Jhp0290 FL and SDS-page

In large scale expression of Jhp0290-17a.a & FL, overnight culture was inoculated into 500ml LB media containing 50μg/ml kanamycin and culture was induced with 100μM IPTG at 37^{o}C with shaking for 2.5hrs. No expression was seen in uninduced sample (Figure 6). Purification of recombinant protein Jhp0290 (-17a.a)

A clarified sample was prepared from a pellet containing Jhp0290 (-17a.a) protein and then loaded on 12% polyacrylamide gel. On the 1st lane marker, uninduced, induced samples and pellet and the supernatant was loaded to know the solubility of the protein. Pellet and supernatant showed a clear band of Jhp0290. Protein from the supernatant was further purified by Talon Resin column. The induced sample showed a clear band and 2nd fraction of elution also showed cleared band. No band showed in uninduced, flow thru, elution 1. In elution 3rd fraction, a similar size band was also seen but not distinct (Figure 7 & 8).

DISCUSSION

In this study, H. Pylori strains J99 and 26696 were used and genes coding for Jhp0290 (-17a.a & FL) were amplified by using Phusion high fidelity DNA polymerase. High-Fidelity DNA Polymerases are important for applications in which the DNA sequence needs to be corrected after amplification [24,25]

Our results showed a clear band on 1% agarose gel after PCR reaction. Genes coding for HP1206 (FL & signal peptide) and HP0305 were successively digested, ligated, and cloned into pET22b & pET21a. Colonies were observed after transformation in E.coli strain (DH5α & BL21(DE3) competent cells, but there was no expression seen at 100μM, 250μM, 500μM, and 1mM concentration of IPTG at 30°C & 37°C with shaking for 3-4hrs. T

he experiment was repeated and the gene coding for J0290-17a.a & FL was amplified by using Phusion DNA polymerase. PCR products were used to clone

Table 3: Amplification and purification of plasmid

Clone	280/260	230/260	DNA
2	**1.87**	1.90	85.17ng/μl
4	1.86	1.83	81.88
6	1.89	1.84	78.77
8	1.86	1.84	75.47

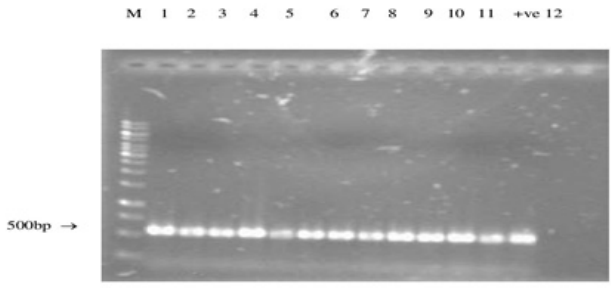

Fig 1: PCR analysis of clones, M= 1Kb Ladder, 1-11 Ladder, 1-11 & 12 represent Jhp0290 (-17a.a) and +ve control = Jhp0290 FL sample were loaded on 1% agarose gel for the verfication of colonies

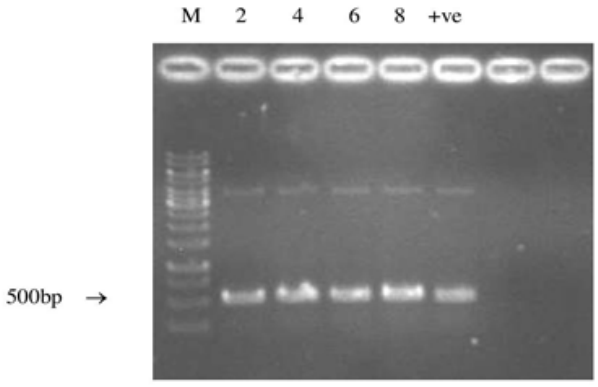

Fig 2- Amplification of insert in recombinant plasmid using PCR, M (1kb Ladder), 2,4 6 & 8 (Jhp0290-17a.a) and +ve control (Jhp0290FL) samples were loaded on 1% agarose gel for the analysis of amplified insert.

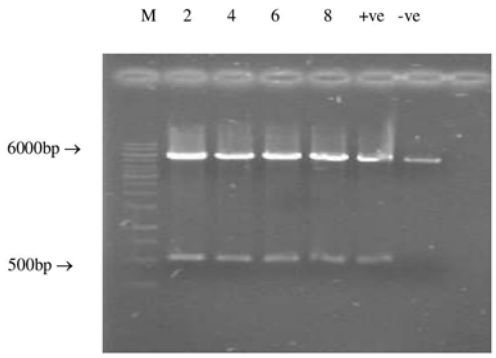

Fig 3- Restriction analysis of clones with XhoI and NdeI restriction enzymes; M=1kb ladder, 2,4,6,8 (Jhp0290-17a.a in PET28b+) +ve (Jhp0290FL in PET28b+) and -ve (PET28b+) samples were loaded on 1% agarose gel

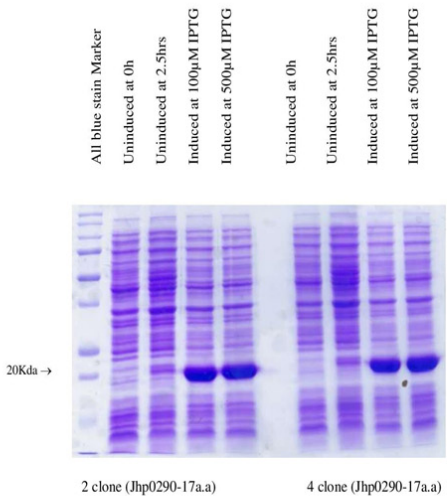

Fig. 4. On the 1st lane, all stain blue marker, 2, 4 clone's uninduced at Oh, uninduced at 2.51rs, induced at 100pM & 500pM IPTG was loaded on 12% separating gel for SDS-page analysis.

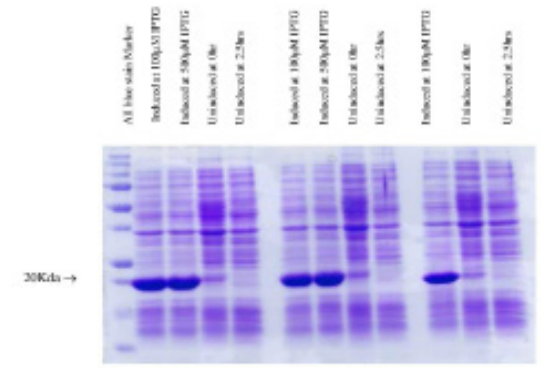

Fig. 5. All blue prestained marker (Biorad) was loaded on l't lane. On successive lanes samples induced with 100pM & 500pM IPTG concentration after 2.5hrs of incubation at 37°C with shaking, loaded on 1% agarose gel. Uninduced samples at Oh & 2.5hrs incubation were also loaded.

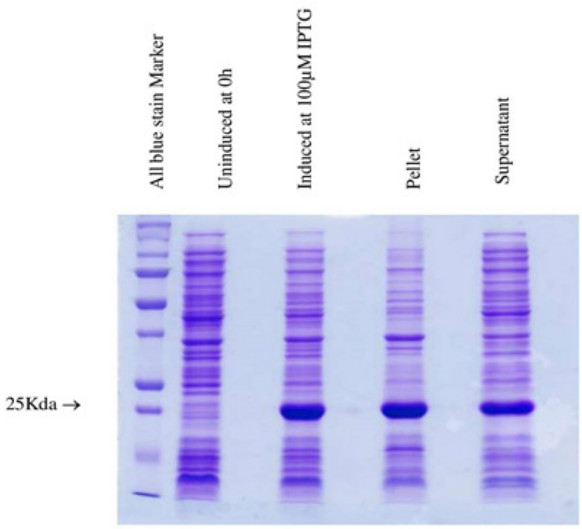

Fig 6- Large scale expression of Jhp0290-17a.a Fl analysis on SDS-Page

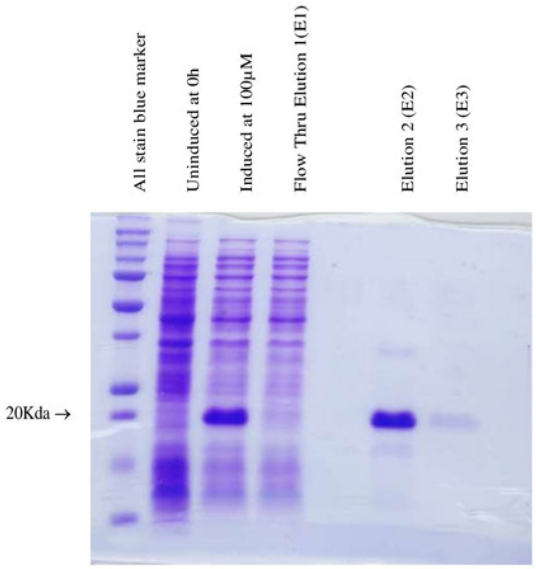

Fig 7- Analysis of protein purification on SDS-Pege

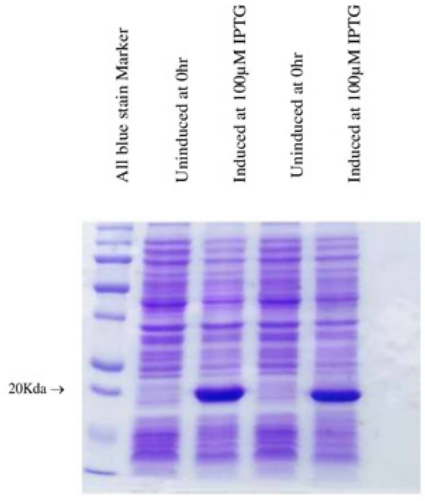

Fig 8- Purification of protein through Talon resin column and analysis on SDS-Page

into pET28b+ vector and transformed into DH5α & BL21 (DE3). Colonies appeared on the LB agar plates with kanamycin (50μg/ml) as a resistance marker strengthening the previous findings by Rourke et al and further experiments were performed to induce the expression of a gene with 100μM to 1mM IPTG concentration[26]. Sometimes, no expression of genes was seen on SDS polyacrylamide gels, and then sequencing of the cloned gene was done by a clinical company.

The results of sequencing showed that there was an error in the gene which ultimately did not induce the expression. The experiments were repeated. Purified plasmid (with 260/280 & 260/230) and the gene coding for Jhp0290 ((-17a.a) were digested with XhoI & NdeI restriction enzymes at 37°C for one hour for plasmid and 2 hours for PCR products that produced blunt ends as carried out earlier by Hamidi et al [27]. Plasmid and PCR product was purified and ligation was performed at 16°C overnight by using T4 DNA ligase. The ligation method was performed at 22°C but showed no colonies on LB agar plate with kanamycin resistance marker because of linear DNA death and showed no resistance to antibiotics. Ligation at 16°C worked well and proceeds further. Colony PCR was performed to verify the selection of good clones and restriction digestion of selected clones to verify the size of the insert. Colony PCR and restriction digestion yielded the same 550bp fragment and the remaining 5.4Kb fragment.

The induction of gene (Jhp0290 -17a.a) expression was noted at 100μM & 500μM IPTG concentration on a small scale. Large-scale expression of the gene was performed at 100μM IPTG concentration and the culture was centrifuged to obtain pellets[28]. Pellets containing Jhp0290 (-17a.a & FL) were dissolved in 1x equilibration buffer and sonicated at 4°C with 30% efficiency for 30 seconds with a one-minute pause for each sonication. Samples were centrifuged to get clarified samples to observe the availability of pellets or supernatant. Jhp0290 (-17a.a) was available in pellets and supernatant but Jhp0290 (FL) was in pellets only. Jhp0290 (-17a.a) was purified as a native protein by using Talon cell thru resin that produced a clear band of highly purified Jhp0290 (-17a.a) protein. The size of protein on SDS polyacrylamide was 22.2Kda and a small faint band was also seen above the clear band.

CONCLUSION

These findings suggest that the expression and release of H.pylori proteins JHP 0290 (FL) and JHP 0290 were different among the strains that alter the host cell response to a variable extent. The development and severity of the disease may be attributed to the level of expression of H.pylori secreted proteins as well as the degree of altered host cell response.

The present research work is an expansion of the existing studies and a significant contribution toward explaining how H. pylori proteins affect the host cell response and provides a framework for future research.

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AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under

Idrees M:	Project design, Critical review, analysis of data
Waqas M:	Conception, literature search and overall supervision
Rahman IU:	Writing up
Ihtesham M:	Statistical analysis
Khan MTM:	Bibliography
Azeem R:	Data collection

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.