Prevalence of Tumor Necrosis Factor Alpha Inducing Protein (*tipα*) Gene of Helicobacter *Pylori* and Its Association with Upper Gastrointestinal Diseases in India

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Research article

Keywords: Helicobacter pylori, Tumor necrosis factor alpha inducing protein, Gastric Cancer, Gastro-duodenal diseases

Posted Date: July 8th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-38351/v1

Version of Record: A version of this preprint was published at 3 Biotech on April 30th, 2021. See the published version at https://doi.org/10.1007/s13205-021-02804-w.

Abstract

Background: *Helicobacter pylori* (*H. pylori*) is known to cause several gastro-duodenal diseases including chronic Gastritis, Peptic Ulcer disease and Gastric Cancer. Virulent genes of *H. pylori* like *cagA*, *vacA* are known to be responsible for the disease pathogenesis. But these virulence genes are not always found to be associated with disease outcome in all populations around the world. Relationship of *H. pylori* with Gastro Esophageal Reflux Diseases is controversial and uncertain. Tumor necrosis factor alpha inducing protein *tipa* is a new discovered virulent gene of *H. pylori* and is an inducer of certain cytokines and chemokines that are responsible for causing stomach cancer. Therefore, we conducted a study which aims to find the prevalence of *tipa* gene in the Indian population and its association with *H. pylori* related upper gastrointestinal diseases.

Results: The current study shows the prevalence rate of *tipa* gene in the Indian population to be 59.9%. Our study has found a significant association ($p < 0.05$) of *tipa* gene with Non Ulcer Dyspepsia (NUD) and also an association of *cagA* and *vacA*s1m1 with Gastritis and Duodenal Ulcer.

Conclusion: Our study demonstrates for the first time the presence of *tipa* as virulent factor of *H. pylori* strain in Indian population isolated from patients suffering from upper gastrointestinal diseases. Further, *tipa* is significantly associated with NUD but not with other upper gastrointestinal diseases in India.

Background

Helicobacter pylori (*H. pylori*) is a Gram negative microaerophilic bacterium that chronically colonizes the gastric epithelium of more than half of the world population and plays an important role in the pathogenesis of several gastro-duodenal diseases such as chronic Gastritis, Peptic Ulcer Disease (PUD) and Gastric Cancer [1]. In India about 53.4–73.5% of the population is infected by *H. pylori* [2]. There is a wide geographical variation in both *H. pylori* related gastro-duodenal diseases as well as distribution of its virulent genes. The prevalence of Gastric Cancer is high in South India as compared to North India while the Eastern and the Northern regions of India have high rates of Duodenal Ulcer (DU) [3]. Role of various virulence factors of *H. pylori*, such as cytotoxin-associated gene Pathogenicity Island (cag PAI), *cagA, vacuolating cytotoxin A* (*vacA*), and *urease* have been studied extensively in gastro-duodenal diseases in India and other parts of the world [4–6]. In East-Asian countries most of the *H. pylori* strains are *cagA*-positive, while 20–40% of isolates from Europe and Africa are c*agA*-negative strains. It is shown that patients infected by *cagA*-positive *H. pylori* have a higher risk of developing PUD or Gastric Cancer compared to those infected with their *cagA* negative counterparts. However, in East Asia, most strains of *H. pylori* have the *cagA* gene irrespective of the disease [7]. In India there are wide geographical differences in the prevalence of *cagA* and *vacA*. About 90% strains from East India [8], 66.6% *H. pylori* strains of North-East India [9] and whereas only 50% of the strains from North India are *cagA* positive (our unpublished data). Also about 80–90% of the strains from East India are *vacA* s1m1 whereas 60.7% of the North East India are *vacA*s1m1 [9, 10]. Therefore, as in the Indian population the strains with s1m1 are pre-dominant. Since there is wide variation in *cagA* and *vacA* prevalence rates in different

geographies in India and also in different parts of the world, it demonstrates that the diseases are multi-factorial. Hence, there is a need to identify other virulent factors which may be responsible for disease causation.

Tumor necrosis factor alpha inducing protein (Tipα) of *H. pylori* is a carcinogenic factor that induces tumor promotion in vitro and in vivo [11–13]. In vitro study by Suganuma et al 2008 shows the binding of *tipα* to surface nuceolin on human Gastric Cancer cell line and that internalization of the *tipα* and nucleolin complex induces tumor progression and epithelial mesenchymal transition in human Gastric Cancer [13, 14]. Tipα is a new NF-κB activating protein of *H. pylori* associated with strong induction of TNF-α in combination with RAS activation. When *H. pylori* infection occurs in the stomach epithelium in which Ras protein is activated or overexpressed, Tipα dimer is assumed to play a carcinogenic role leading to Gastric Cancer [15]. However, if *H. pylori* infection occurs in the stomach epithelium without activated Ras protein, Tipα dimer will probably not produce Gastric Cancer, but only inflammation (Gastritis and Gastric Ulcer). Although activated Ras protein is not often found in human stomach cancer, it is now possible to conceive a new regulatory mechanism of Ras protein with the let-7 microRNA [15]. Though there is a published sequence of one of the Indian strains of *H. pylori* (India 7 strain (NC_017372), (HPIN_03690) showing the presence of this gene, there are no studies on Tumor necrosis factor inducing protein (Tipα) from India. Tipα, which induces the expression of variety of pro-inflammatory cytokines including TNF and IL-1, is over-expressed in gastric mucosa exposed to *H. pylori* [13]. Although, India constitutes around one-sixth of the world population, there are no studies on the prevalence of *tipα* in *H. pylori* related gastrointestinal diseases. This prompted us to study the prevalence of the *tipα* genes from *H. pylori* strains isolated from various parts of India and its association with *H. pylori* related gastrointestinal diseases.

Results

Prevalence of *tipα* gene in the Indian population

We analyzed 267 clinical *H. pylori* isolates for the prevalence of *tipα* gene and its association with *cagA* and *vacA* gene using PCR assay. The *H. pylori* isolates included in the study were of various gastrointestinal diseases which are Gastritis (n = 80), Duodenal Ulcer (DU) (n = 77), Non Ulcer Dyspepsia (NUD) (n = 51), Gastric Ulcer (n = 7), Gastro-Esophageal Reflux Disease (GERD) (n = 32) and Controls (n = 20). The genotyping of *tipα* was done using primer as shown in Table 4 which gave an amplicon size of 168 bp (Fig. 1). The prevalence rate of *tipα* gene was found to be 59.9% (160 out of 267) (95% CI: 54.36–100%) in the Indian population. However, 100% (27/27) of the *H. pylori* strains isolated from East India are *tipα* positive followed by 90% (9/10) in South India, 75.3% (49/65) in North India and only 45.4% (75/165) in North-East India (Table 1).

Genotypic status of *cagA* and *vacA* gene

Multiplex PCR was done for detection of *cagA* and *vacA* gene in all *H. pylori* isolates. (Fig. 2). The *cagA* gene was detected in 79.8% isolates (213/267) (95% CI: 53.7–97.6%) and rest 20.3% isolates (54/267)

showed absence of *cagA* gene which was further confirmed using empty site PCR (Table 1). Region wise more than 90% of the *H. pylori* isolates are *cagA* positive in North East India 93.9% (155/165) and South India 90% (9/10) followed by East India showing prevalence of 74% (20/27). However surprisingly only 44.6% (29/65) of the North Indian strains are *cagA* positive (Table 1).

The genotype of different *vacA* alleles examined in overall isolates were found to be s1m1 in 75.3% (201/267), s2m2 in 16.8% (45/267) and s1m2 in 7.9% (21/267) isolates (Table 1). *vacA* s1m1 allele is found to be positive in more than 90% of the *H. pylori* isolates from North East and South India. The *H. pylori* isolates from East India shows that 55.5% (15/27) strains are s1m1, 18.5% (5/27) are s1m2 and only 25.9% (7/27) of the strains are s2m2. However, the strains from North India shows that 40% (26/65) are s1m1, 16.9% (11/65) are s1m2 while 43% (28/65) are s2m2 (Table 1).

Table 1
Prevalence of *tipα, cagA and vacA* gene in *H. pylori* isolates from different regions of India.

Isolates from different states	**Total no. of isolates**	***tipα* positive isolates (%)**	***tipα* negative isolates (%)**	***cagA* positive isolates (%)**	***cagA* negative isolates (%)**	***vacA* s1m1 (%)**	***vacA* s2m2 (%)**	***vacA* s1m2 (%)**
North East India	165	75 (45.4)	90 (54.5)	155 (93.9)	10 (6.1)	151 (91.5)	10 (6.06)	4 (2.42)
North India	65	49 (75.3)	16 (24.6)	29 (44.6)	36 (55.4)	26 (40)	28 (43)	11 (16.9)
South India	10	9 (90)	1 (10)	9 (90)	1 (10)	9 (90)	0 (0)	1 (10)
East India	27	27 (100)	0 (0)	20 (74)	7 (26)	15 (55.5)	7 (25.9)	5 (18.51)
Total	**267**	**160 (59.9)**	**107 (40.1)**	**213 (79.8)**	**54 (20.3)**	**201 (75.3)**	**45 (16.8)**	**21 (7.9)**

Association of *tipα*, *cagA*, *vacA* with different disease outcomes

Table 2 shows association of *tipα*, *cagA* and *vacA* with different disease outcomes. Our study shows the presence of *tipα* gene in *H. pylori* isolated from patients suffering from various upper gastrointestinal diseases – 41.3% Gastritis (33/80), 84.38% Non Ulcer Dyspepsia (43/51), 58.4% Duodenal Ulcer (45/77), 42.9% Gastric Ulcer (3/7), 75% GERD (24/32). The association of *tipα* gene was found significant only in Non Ulcer Dyspepsia (NUD) with respect to the controls ($p < 0.05$) (Table 2). Significant association of

cagA virulent gene was found in Duodenal Ulcer ($p < 0.01$) and Gastritis ($p < 0.001$). *vacA*s1m1 allele was also found to be significantly associated with Duodenal Ulcer ($p < 0.05$) and Gastritis ($p < 0.05$).

Table 2
Detection and distribution of *H. pylori* and virulent genes in patients with various gastrointestinal disease outcomes.

	Control (%) n = 20 (7.5)	Gastritis (%) n = 80 (29.9)	GERD (%) n = 32 (12)	NUD (%) n = 51 (19.1)	DU (%) n = 77 (28.8)	Gastric Ulcer (%) n = 7 (2.6)	Total (%) n = 267 (100)
cagA positive	13 (65)	74 (92.5)*	16 (50)	37 (72.5)	68 (88.3) *	5 (71.4)	213 (79.7)
cagA negative	7 (35)	6 (7.5)	16 (50)	14 (27.5)	9 (11.7)	2 (28.6)	54 (20.3)
tipα positive	12 (60)	33 (41.3)	24 (75)	43 (84.3) *	45 (58.4)	3 (42.9)	160 (59.9)
tipα negative	8 (40)	47 (58.8)	8 (25)	8 (15.7)	32 (41.6)	4 (57.1)	107 (40.1)
*vacA*s1m1	12 (60)	74 (92.5) *	14 (43.8)	31 (60.8)	65 (84.4) *	5 (71.4)	201 (75.2)
*vacA*s2m2	4 (20)	6 (7.5)	14 (43.8)	12 (23.5)	7 (9.1)	2 (28.6)	45 (16.9)
*vacA*s1m2	4 (20)	0 (0)	4 (12.5)	8 (15.7)	5 (6.5)	0 (0)	21 (7.9)
*p < 0.05							

Association of *tipα* with *cagA* and *vacA* gene

In our study we found 56.8% (121/213) *cagA* positive and 72.2% (39/54) *cagA* negative showed presence of *tipα* gene but the association was found to be not significant (Table 3). We found that 95.2% of *vacA*s1m2 also shows the presence of *tipα* and the association is found to be significant ($p < 0.05$). 71.1% of *vacA*s2m2 and 53.7% of *vacA*s1m1 shows the presence of *tipα* but the association was not significant.

Table 3
Association of *vacA*, *cagA* and *tipa* in *H. pylori* strains

	Total *H.pylori* isolates (%) n = 267	*cagA* positive (%) n = 213	*cagA* negative (%) n = 54	*vacA*s1m1 (%) n = 201	*vacA*s2m2 (%) n = 45	*vacA*s1m2 (%) n = 21
tipa Positive	160 (59.9)	121 (56.8)	39 (72.2)	108 (53.7)	32 (71.1)	20 (95.2) *
tipa negative	107 (40.1)	92 (43.1)	15 (27.7)	93 (46.2)	13 (28.9)	1 (4.7)
*p < 0.05						

Discussion

There are several studies which have shown, that the incidence and / or severity of gastrointestinal diseases related to *H. pylori* may vary geographically [16]. Further, there are several reports for the presence of different strains of *H. pylori* with different degree of virulence indicating variation in the distribution of different virulent genes of *H. pylori* in different populations [17–19]. Several Indian studies show that *cagA* gene was found at a high frequency of 86% of the *H. pylori* strains and this virulence marker was found at almost equal frequencies in strains from DU patients (90.6%) and NUD patients (82%), indicating that the prevalence of the *cagA* gene cannot be considered as a key virulence marker for determination of the clinical status of the host [10, 20]. This is very similar to the results in our present study, where we found the prevalence rate of 79.8% for *cag A* positivity among *H. pylori* related gastrointestinal diseases. We found large geographical differences in the prevalence rates in different parts of India. We found that the prevalence of *cagA* in North India, South India, North-East India were 44.6%, 90% and 93.9% respectively. *cagA* was significantly associated ($p < 0.05$) with Gastritis and DU, where its prevalence was 92.5% and 88.3% respectively compared to prevalence rate of 65% (13/20) among controls. On the other hand, the prevalence of *cagA* gene among GERD, NUD and Gastric Ulcer were 50%, 72.5% and 71.4% respectively but was not significant. Our results are in line with the findings and observations of other studies from India which show a high *cagA* prevalence among DU and Gastritis patients. Prevalence was also high among NUD and PUD patients but they did not reach significance. The prevalence of *cagA* phenotype was also lower in patients with GERD as has been the observation of other studies as well [21].

We also found the overall prevalence rate of 75.2% for the *vacA*s1m1 allele was highest followed by *vacA*s2m2 (16.9%) and *vacA*s1m2 (7.9%). This data is in accordance to previous studies having about 70% s1m1 allele in Indian *H. pylori* isolates [10]. We found significant association ($p < 0.05$) of the virulent allele s1m1 with Gastritis and Duodenal Ulcer but not with others viz. GERD, DU and Gastric Ulcer. Our present observations are in line with the other studies from India [20]. Although there is geographical variation in the prevalence of *vacA*s1m1 with 91.5% in North East India, 90% in South India, 74% in East

India. In North India, the prevalence of s1m1 is 40% where as 43% is s2m2 which is similar to reports from Kauser et al 2005 from Ladakh, India where they found that 60% of the strains have s2 genotype [22].

We for the first time in the world analyzed the prevalence of *tipa* gene among the *H. pylori* strains and its association with virulent genes *cagA*, *vacA* gene in various gastrointestinal diseases. Our study shows that the overall prevalence of *tipa*, among the Indian *H. pylori* isolates is 59.9%. NUD has been to be significantly associated with tip*a* with a prevalence rate of 86.3% compared to the prevalence of 60% among controls. The prevalence rate were similar to NUD (86.3%) and GERD while the prevalence rates were much lower in Gastritis (41.3%), DU (57.1%) and Gastric Ulcer (42.9%). The highest prevalence rate of *tipa* gene was found in 95.6% (22/23) *vacA*s1m2 positive *H. pylori* strains indicating a strong correlation between them. Another study has also reported increased mucosal IL-18 mRNA expression in *vacA*s1m2 allele causing increase in the risk of gastro-duodenal disease although no significance was found [23]. It has been found that Gastric Cancer patients infected with *tipa* positive strains of *H. pylori* produce significantly higher amounts of TNF-*α* than patients with chronic Gastritis and that TNF-*α* induced inflammatory response plays a significant role in the development of Gastritis and Gastric Cancer associated with *H. pylori* infection [24].

Our study also demonstrates the significant association of *tipa* gene with Non Ulcer Dyspepsia (NUD). Non Ulcer Dyspepsia refers to heterogeneous and broad range of chronic upper abdominal symptoms which are widely shared with different gastrointestinal disorders. Therefore, NUD is generally diagnosed when other frequent gastrointestinal diseases are excluded, and upper endoscopy ruled out macroscopic lesions - i.e., Gastritis or Peptic ulcer. Such a condition is frequently encountered in clinical practice, its prevalence being close to 20%-30% in the general population and probably predates the occurrence of Gastritis and PUD. To date, no conclusive data have been reported on the role of *H. pylori* infection on these dysfunctions. A Cochrane Meta-analysis of 17 trials with 3,566 patients showed that there was a 10% (95% CI: 6–14) relative risk reduction of dyspepsia following *H. pylori* eradication as compared to placebo, with a NNT of 14 (95% CI: 10–25). *H. pylori* infection may cause dyspeptic symptoms in NUD through other mechanisms such as: (1) alterations of gastric acid secretion; (2) persistent and active inflammation of gastric mucosa; and (3) post-infective changes in gastroduodenal mucosa. Our study raises a possibility of *tipa* being the link between *H. pylori* and NUD. This merits a further larger studies to comprehensively understand this possibility [25].

In summary, our results revealed, for the first time in the world that the prevalence rate of *tipa* gene is 59.9% (160 out of 267) among the Indian population. There is a highly significant association between virulent gene *cagA* and *vacA*s1m1 alleles with Duodenal Ulcer and Gastritis. We found a significant association of *tipa* gene with Non Ulcer Dyspepsia (NUD). Further, larger studies will be required to determine the clinical relevance of Tip*a* as a clinical parameter in NUD and other *H. pylori* related gastro-intestinal diseases.

Conclusion

The present study concludes for the first time that the Indian population has 59.9% prevalence rate of *tipα* gene. Our study also shows that there is a strong association of *tipα* with *vacA*s1m1 and *cagA* gene with Duodenal Ulcer and *tipα* gene with Non Ulcer Dyspepsia (NUD) disease.

Abbreviation

Non Ulcer Dyspepsia (NUD)

Helicobacter pylori (*H. pylori*)

Peptic Ulcer Disease (PUD)

Duodenal Ulcer (DU)

vacuolating cytotoxin A (*vacA*)

cytotoxin-associated gene Pathogenicity Island (cag PAI)

cytotoxin-associated gene A (cagA)

Tumor necrosis factor alpha inducing protein (Tipα)

Gastro-Esophageal Reflux Disease (GERD)

Methods

Collection of *H. pylori* specimen

A total of 267 *H. pylori* positive isolates across different parts of India were included in the study-North India (n=65), South India (n=10), East India (n=27) and North-East India (n=165). The *H. pylori* strains were isolated from patients suffering from various upper gastro intestinal tract diseases (n=247), controls (n=20). Controls were defined as patients who were suffering from disease condition not related to *H. pylori* related gastro-duodenal diseases like Diarrhoea, Celiac disease, Eosinophilic Gastritis etc who underwent endoscopy as a part of standard treatment protocol.

Culturing of *H. pylori* and DNA extraction

Brucella broth containing the biopsy samples were vortexed in laboratory for 2 min and 200 µl of the mixture was streaked onto on brain heart infusion agar plates with charcoal (BHIA; Becton Dickinson, Sparks, MD, USA) which was supplemented with 5% Horse Serum; 0.4% IsovitaleX (Becton Dickinson, Sparks, MD, USA) and antibiotics such as Amphotericin B (8µg/ml), Trimethoprim (5µg/ml) and Vancomycin (6µg/ml) and incubated under microaerophilic condition (5% O_2; 10% CO_2; 85% N_2) in a double gas incubator (Heracell 150i) at 37°C. *H. pylori* were identified on the basis of their typical water droplet like morphology and urease, oxidase and catalase test result. The genomic DNA was extracted

using standard protocol of CTAB [26] method with phenol/chloroform and isopropanol precipitation as described elsewhere as well as kit based method (QIAamp DNA Mini Kit) as per the manufacturer protocol and was stored at -20^{o}C at Amity University, Noida and NICED, Kolkata.

Molecular characterization of genotypic status by PCR Amplification

Genotyping of *cagA* and *vacA* gene was performed using multiplex PCR assay using 2.5 pmol of primers VAG-F and VAG-R, 25 pmol of primers VAI-F and VAI-R, 10 pmol of primers cag5c-F and cag3c-R as shown in Table 4 [27].

Genotyping of *tipa* gene was carried out using simplex PCR (Table 4). The PCR was performed with the final volume of 20 µl holding 10 ng of bacterial genomic DNA, 20 pmol of each primer, 0.25 mM of each dNTPs (Bangalore Genei), 1U of Taq DNA polymerase (Bangalore Genei) in standard PCR buffer (Bangalore Genei) containing 1.5 mM $MgCl_2$ and were amplified according to cycling conditions: 35 cycles at 94^{o}C for 1 minute, 60^{o}C for 1 minute and 72^{o}C for 1 minute with final extension of 10 min at 72° C in Eppendorf (Vapo- protect). Amplified products of multiplex and simplex PCR were then analyzed by using 2% and 1% agarose gel respectively in 1X TAE buffer containing 0.05% µg/ml EtBr followed by screening running under UV trans-illuminator (Tarsons).

Statistical Analysis

The analysis of data was carried out using Fisher exact test and the data was statistically significant ($P < 0.05$).

Table 4
Primers used for the genotyping of *H. pylori* strains.

Genes	Primer	Nucleotide sequence (5´ to 3´)	Amplicon size in bp	References
cagA (5´ end)	cag5c-F	GTTGATAACGCTGTCGCTTCA	350	[27]
	cag3c-R	GGGTTGTATGATATTTTCCATAA		
vacA s1/s2	VAI-F	ATG GAA ATA CAA CAA ACA CAC	s1;259 s2;286	[27]
	VAI-R	CTG CTT GAA TGC GCC AAA C		
vacA m1/m2	VAG-F	CAATCTGTCCAATCAAGCGAG	m1;567 m2; 642	[27]
	VAG-R	GCGTCAAAATAATTCCAAGG		
tip-α	Tip-α-F	GTT TAT CCA AAT GAC ACA GCC CAT	168	This study
	Tip-α-R	CAC CGC TTG ATT GTC TAA GGA CAT		

Declarations

Ethics approval and consent to participate: This study was approved by institutional ethical committee of Amity Institute of Biotechnology, Noida, U.P, dated 2-12-2015 and National Institute of Cholera and Enteric Diseases, Kolkata (A-1/2016-IEC, dated 22-12-2016). Written informed consent was taken.

Consent to Publish: Not Applicable

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests: The authors declare that they have no competing interests

Funding: This study has been funded by ICMR (5/13/16/2015/NCD-III)

Acknowledgement: We thank Amity University for providing the infrastructure and support to carry out the work.

Authors' contributions: RD and SB have given the concept of association between Tip α and Gastric Cancer. RD and AKM have finalized the manuscript. SM, SP, BCK and AC have done the experiments. SM has analyzed the data and written the manuscript. AKM has provided the samples and revised the manuscript. KD has conceptualized the idea clinically and critically corrected the manuscript.

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Figures

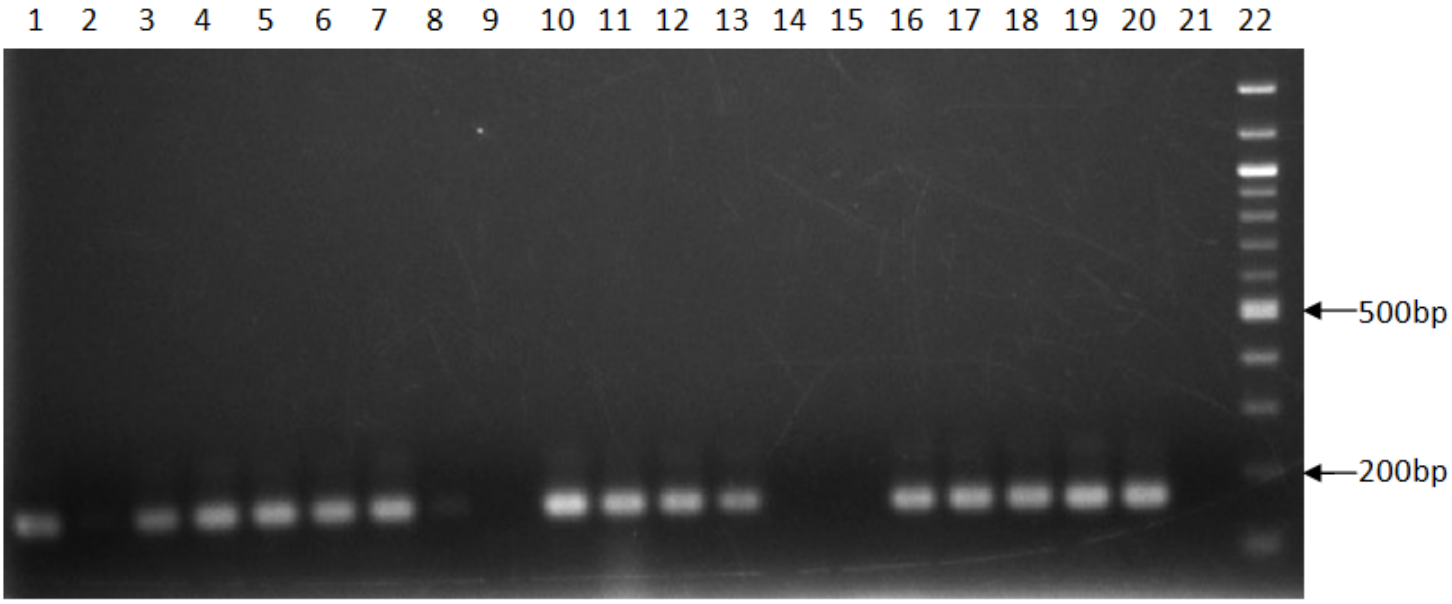

Figure 1

DNA amplification of tipα gene of 168bp from H. pylori strains. Lane1- positive control, 26695; Lane 2-21 H. pylori clinical isolates and Lane 22- Marker (100bp).

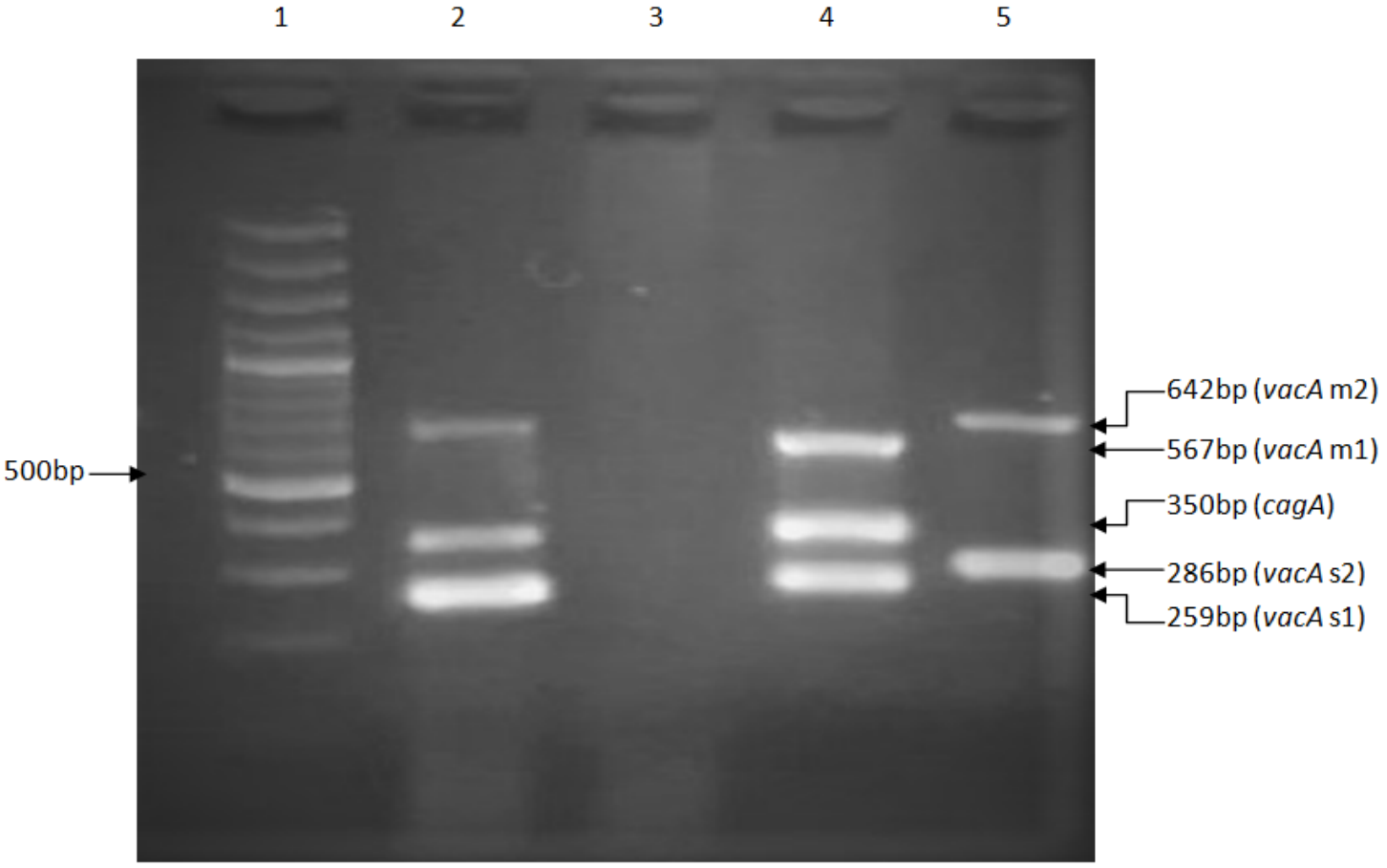

Figure 2

Multiplex PCR of vacA s1/s2 and m1m2 and the cagA gene of H. pylori strains. Lane 1, 100bp marker; lane 2 positive control, 26695, lane 3 negative control, lane 4 H. pylori clinical isolate cagA and vacA (s1m1), lane 5 H. pylori clinical isolate vacA (s2m2).