Helicobacter pylori Virulence Regulatory Network: Insights into the Host-Environment and Pathogen Interactions

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ABSTRACT

Helicobacter pylori (*H. pylori*) has evolved significant regulatory mechanisms in order to acclimatize in extreme gastric environment of human beings. The virulence machinery of H. pylori is complicated as virulence factors of pathogen not only interact with transcription and translational machinery of host, but also are involved in the progression and development of the disease. The present study is an effort to model virulence mechanism in H. pylori, particularly ferric uptake regulator (FUR) under acidic and iron (Fe) depleted conditions, as well as its effects on the well known virulence factors cytotoxin-associated gene A (cagA) and vacuolating cytotoxin A (vacA) gene. The virulence regulatory network of cagA and vacA is modeled based on an asynchronous kinetic logic formalism introduced by René Thomas. The cagA-vacA virulence regulatory network is then elaborated qualitatively to obtain insights into H. pylori induced pathogenesis. The findings have revealed the significant regulatory pathways through which H. pylori spreads infection to the gastric cells, and also verified that cagA is associated with acute gastritis while vacA is involved in vacuolation, apoptosis and atrophy. Interestingly, both cagA and vacA were found to modulate each other virulence potential which ultimately leads to the state of chronic gastritis; which in turn drives the pathway smoothly towards gastric adenocarcinoma via the formation of pre-malignant lesions. The proposed strategy can be extended to understand the mechanism of other similar bacterial infections and disease progression. It will also help in the prioritization of potential therapeutic targets to control such serious infections.

Key words: Helicobacter pylori, CagA, Virulence, Adenocarcinoma, Regulatory networks.

1. INTRODUCTION

Helicobacter pylori (*H. pylori*) affects more than 50% of the world's population and the infection is more prevalent in developing countries (~90%) in comparison to the developed world (~20%). However, in most of the cases, the infection remains asymptomatic (Blanchard, et al., 2004). *H. pylori* infection is the primary cause of chronic gastritis which is recognized as one of the risk factor for the development of gastric carcinoma. Besides other gastric pathologies like gastric and peptic ulcerations and lymphomas, *H. pylori* associated gastritis also leads to gastric cancer (about 70% globally) (Peek and Blaser, 2002). Gastric cancer is amongst the most common cancers and second leading cause of cancer related deaths, worldwide (Ferlay, et al., 2010). Therefore, the pathogen is classified as Class I carcinogen by WHO (Humans, 1994). Inside the host, *H. pylori* can live in extreme conditions such as gastric acidity and iron limitation. In order to cope with such conditions, the pathogen has evolved specific but rather limited set of transcriptional regulators (Salama, et al., 2000, Baltrus, et al., 2009). These transcriptional regulators are superior to their homolog proteins found in other bacteria in terms of their additional roles besides their traditional functions. For example, ferric uptake regulator (Fur) is one of the most important global transcription regulator (Delany, et al., 2001, Delany, et al., 2001, Alamuri, et al., 2006, Gilbreath, et al., 2012, Pich, et al., 2012) which facilitates bacterial pathogen to acclimatize in acidic and iron limiting conditions (Bereswill, et al., 2000). In most of the bacterial species, including *H. pylori*, the Fur exists in the form of a complex (Fe-Fur), which binds to the promoters of iron uptake genes and regulates their transcription. However, Fur in *H. pylori* has acquired the ability to bind the promoter of other host genes independent of the complex (Apo-Fur) and regulates the transcription process (Bereswill, et al., 2000, Delany, et al., 2003, Ernst, et al., 2005). This dual property of Fur broadens its regulatory control on the expression of genes, which in turn equip the bacterium to adapt in environment with low iron levels.

This study focuses on the dual property of Fur to regulate the genes which can help *H. pylori* to survive and colonize in acidic environment and nutrient limited conditions for several years, ultimately leading to inflammatory responses and resulting in significant clinical consequences like ulceration and gastric neoplasm (Blaser & Parsonnet, 1994). It is, therefore, necessary to understand the biology of *H. pylori* infection, not only because it is causative agent of adenocarcinoma but also can be a representative model of effects of chronic inflammation on gastric mucosa. (Blaser & Parsonnet, 1994).

Virulence machinery of *H. pylori* is quite complicated and

various interacting factors (virulence factors and host factors) are involved in the progression and development of disease (Atherton, 1998, Höcker & Hohenberger, 2003, Kusters, et al., 2006). This developmental behavior and the dynamics of the pathogenesis vary in the presence of different environmental cues (such as low pH, iron availability etc.). No doubt, the interplay of various key players in the pathogenesis of *H. pylori* complicates the prediction of exact virulence mechanism. Recent advances and development of high-throughput techniques in computational and system biology enhanced our understanding of such complex biological systems. These techniques have greatly contributed in the accumulation and growth of quantitative and qualitative biological information, which leads to the prediction of biological interfaces at different levels (Saadatpour & Albert, 2013). In order to target single outcome or behavior, it is necessary to identify the virulence factors and re-construct the whole or representative virulence regulatory network (VRN) (Karlebach & Shamir, 2008).

The kinetic logic based method builds biological regulatory network (BRN) exploiting threshold response. The biological entities (genes or proteins) interact with each other either positively or negatively. In other words, certain concentration of a particular entity can either increase or decrease the activation of other entity. Thus two types of biological regulation i.e. either positive regulation or activation and negative regulation or inactivation are the essential interactions between genes or/and products of BRN. Both types of biological regulation are a function of sigmoidal curve (Ahmad, et al., 2012).Thus our study studies the pathogenic nature of H. pylori in gastric carcinogenesis pertaining to the regulatory expression of cagA and vacA, this regulatory expression along with other associated factors is referred to as virulence regulatory network (VRN), which has been constructed by logical modeling based on Kinetic logic formalism and with the help of existing experimental evidences. The results generated may show various regulatory pathways in the form of a state graph. These pathways form a network in which pathological outcomes (states) will be produced because we expect differential expression of virulence factors under different environmental stresses. These states may cross each other and regulate other states to produce an outcome; in case of *H. pylori* that is gastric carcinogenesis.

2. METHODOLOGY

2.1. Network modeling

In network modeling, the components are shown as nodes, while interactions are represented by edges. These interactions could be of two types; positive and negative symbolizing activation and inhibition of the nodes, respectively. In biological systems, large number of components including genes, proteins and their corresponding levels leads to a complex interaction networks. Translating the structure and dynamics of such networks is the primary step in understanding the overall behavior of the cell (Karlebach & Shamir, 2008). To target a single outcome and behavior, such network modeling techniques can be modified to include specific nodes and interactions and study the respective structural and dynamical analysis to uncover the underlying specific biological organization.

The network modeling strategies can be broadly classified in two types; dynamic modeling and discrete modeling. Dynamic modeling takes nodes as molecular species and considers the effect of time on the population levels of species. It observes and describes how the rate of population levels changes with respect to time. These models are illustrated by set of differential equations and are more reliable in extracting the dynamical behavior of biological system. Whereas discrete modeling e.g. Boolean models, finite state logical models and Petri nets are helpful in providing qualitative description with few or no parameters (Saadatpour & Albert, 2013).

René Thomas in 1970 proposed Boolean logic method for the discrete modeling of biological regulatory network (BRN) with the corresponding qualitative modeling of dynamical behavior of system. However, René Thomas rendered this method limited because this method uses only two levels 0 and 1, which is not sufficient to encompass other types of problems. Subsequently René Thomas modified the Boolean logic to Kinetic logic and demonstrated its practical reliability by applying to different gene regulatory networks, which is adopted successfully in our previous studies (Ahmad, et al., 2012, Paracha, et al., 2014).

2.2. Prediction of *H. pylori* Virulence Regulatory Network (VRN)

The general overview of pathogenesis of *H. pylori* was performed through extensive literature survey and consulting through virulence factor databases such as (VfDB (Chen, et al., 2005) and VirDB (Mazzoleni, et al., 2003), which includes the identification of related virulence factors (genes, proteins and mRNAs), nature of interactions between them as well as the global regulations by transcriptional factors (Figure 1).

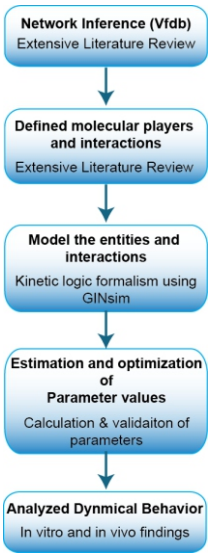

Figure 1. Outline of the Proposed Methodology. The workflow represents the schematic representation of the methodology used in the modeling of cagA-vacA VRN. The stages in this study overlap at variouspoints, which helps in refining (model reduction) the outcome of predicted VRN.

2.3. Discrete Modeling of Proposed CagA-VacA VRN

The cagA-vacA VRN was modeled using Gene Interaction Network simulation (GINsim), a computational tool that is used for the modeling and simulation of gene regulatory networks. GINsim is a java-based platform, which can be employed in qualitative modeling and analysis of biological regulatory networks. It includes graphical user interface (GUI) which helps user to draw and edit the predicted regulatory network (Gonzalez, et al., 2006). It also allows to stabilize the regulatory graph with the inclusion of logical parameters (observations) and a simulation engine to help establish qualitative dynamic behavior of the corresponding VRN (Gonzalez, et al., 2006). The tool is freely available (http://www.ginsim.org/) and is based on well-known logical formalism first introduced by René Thomas (Gonzalez, et al., 2006).

This logic known as kinetic logic is based on following definitions (Gonzalez, et al., 2006):

- **Logical Regulatory Graph:** This type of graph illustrates the regulatory interaction between genes and their products.
- **State Transition Graph:** This type of graph constructs the relative dynamical behavior from the given regulatory graph/network for the presumed initial (starting) states.

2.4. Construction of cagA-vacA VRN as Logical Regulatory Graph

With the graphical user interface (GUI) of GINsim, the regulatory graph can be created, edited and saved in "ginml" extension. Within the workspace of GINsim platform, genes can be added and interactions (activation and inhibition) can be drawn by selecting the option 'node' and 'arc' from drop down menu under the tab 'edit'. Following nodes were added into the cagA-vacA VRN; two virulence factors, i) cagA ii) vacA, one global regulator iii) fur and two environmental factors iv) low pH (acid) and v) iron limitation (Fe) as shown in Figure 2. The interactions between genes were created by adding an arc (edge) and drawing it from the node (activator/inhibitor) to another node (to be 'activated'/'inhibited' node). Modeling attributes can be added by clicking on a desired node; Id, name, checking or unchecking the input status and maximum expression level may be added. Here, all nodes were attributed maximum expression value "1" while all edges were assigned threshold value "1".

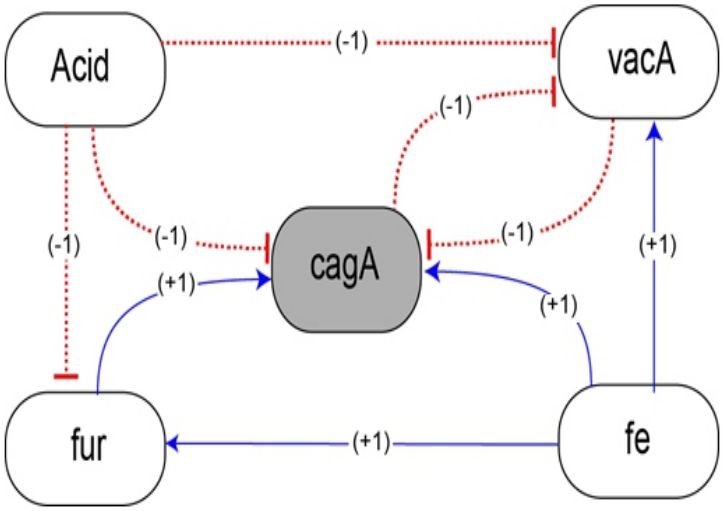

Figure 2. Logical regulatory graph of cagA-vacA Virulence Regulatory Network. The VRN shows the regulation of two important *H. pylori* virulence factors, cagA and vacA in a high acidic gastric niche and iron limiting conditions. Under simultaneous conditions of low pH and iron limitated cagA, vacA and fur are repressed. As the concentration of Fe increases, it binds to the Fur that inducts the positive regulation of cagA and vacA. CagA and VacA modulate each other activity by selectively inhibiting one another. The signs "+" (blue) and "-" (red) represent activation and inhibition, respectively. The integer "1" represents the qualitative threshold of an interaction.

2.5. Computation and Optimization of Parameter Values

For the prediction of dynamical behavior of cagA-vacA VRN, it is necessary to define parameter values or logical values for each node in VRN. For each node, the corresponding logical parameter allowed the qualitative specification of the effects of any combinations of incoming interactions (Gonzalez, et al., 2006). The total number of K parameters for each node/entity is calculated by a formula 2n where 'n' represents the number of interactions (either negative or positive) coming towards node. In GINsim, the K parameters of desired node can be added by clicking on the individual and combined interactions coming towards a particular node. The general format of a K parameter is Kentity,{resources}, where resources are the set of activators (positive regulator) when they are present (at level 1) and inhibitors (negative regulator) when they are absent (at level 0) at any instant of time. The K parameters are defined by giving either a value '0' or "1" (representing expression levels). The K parameter Kentity, {resources}=1, increase the expression of the entity to 1 when its present level is 0. Similarly, The K parameter Kentity,{resources}=0, decreases the expression of the entity to 0 when its present expression level is 1. If the entity level is equal to its parameter value then entity may not evolve. The complete sets of parameters of all entities along with their experimental evidences are given in **Table 1.**

Table 1. Logical parameters of cagA-vacA VRN. The logical parameters (K parameters) adjusted for each entity and employed in GINsim to modelled the cagA-vacA VRN.

Entity	No. of Interactions (n)	Total No. of possible K parameters (2^n)	K parameters associated with entity	Reference studies
CagA	4	2^4=16	$K_{cagA}\{\ \}=0$, $K_{cagA}=0$, $K_{cagA}\{Acid,Fe\ \}=1$, $K_{cagA}\{Acid,Fur\}=1$, $K_{cagA}\{Acid,VacA\}=0$, $K_{cagA}\{Acid,Fe,Fur\}=1$, $K_{cagA}\{Acid,Fe,VacA\}=1$, $K_{cagA}\{Acid,Fur,VacA\}=0$, $K_{cagA}\{Acid,Fe,Fur,VacA\}=1$, K_{cagA}(Ferlay, *et al.*)=1, $K_{cagA}\{Fe,Fur\ \}=1$, $K_{cagA}\{Fe,VacA\ \}=0$, $K_{cagA}\{Fe,Fur,VacA\}=1$, $K_{cagA}\{Fur\ \}=1$, $K_{cagA}\{Fur,VacA\ \}=0$, $K_{cagA}\{VacA\ \}=1$	(Merrell, *et al.*, 2003, Gupta, *et al.*, 2011, Raghwan & Chowdhury, 2014)
VacA	3	2^3=8	$K_{vacA}\{\ \}=0$, $K_{vacA}[Acid]=0$, $K_{vacA}\{Acid,CagA\}=0$, $K_{vacA}\{Acid,Fe\}=1$, $K_{vacA}\{CagA\}=0$, $K_{vacA}\{CagA,Fe\}=0$, K_{vacA}(Ferlay, *et al.*)=1, $K_{vacA}\{Acid,CagA,Fe\}=1$	(Merrell, *et al.*, 2003, Bury-Mone, *et al.*, 2004, Gupta, *et al.*, 2011, Raghwan & Chowdhury, 2014)
Fur	2	$2^{2=}4$	$K_{Fur}\{\ \}=0$, K_{Fur} (Ferlay, *et al.*)=0, $K_{Fur}\{Acid\ \}=0$ $K_{Fur}\{Acid,Fe\}=0$	(Raghwan & Chowdhury, 2014)

2.6. Construction of State Graph

After cagA-vacA VRN was constructed the state graph was generated by running a command 'run simulation' under the 'actions' menu. The interface of state transition graph includes detailing of initial states, choosing between synchronous and asynchronous construction strategy. It allows user to choose either width or depth first search algorithm for the exploration of state graph extracted from the stabilized and parameterized regulatory graph. The construction strategy chosen for cagA-vacA VRN was asynchronous and depth first search algorithm was selected. Next limits on depth and number of states can be optimized; however, this option was not used in our cagA-vacA VRN. After the generation of state transition graph, stable state(s) were identified. In generated state graph, a stable state or sink, depicts the entire system assembly, terminal point and where it cannot progress to the subsequent state, while starting state confers to a state from which all initial trajectories are originated (Ahmad, et al., 2012). States other than stable state(s) leading to and terminating at sink(s) are called trajectories. It is property of a system to achieve stability at any moment, the system is disturbed from its stable state or sink it falls back or moves to another stable state.

2.7. Analysis of the State Graph

The state transition graph using GINsim simulation tool usually comes out as complex mesh of network, which is usually hard to analyze. This state transition network can be sorted out manually by first separating out stable state(s) and then logically arranging the remaining trajectories. The Graphviz tool is graph visualization software, which represents structural data as diagrams of predicated graphs and networks in a systemic and hierarchical manner (Ellson, et al., 2002).This tool is available freely on http://www.graphviz.org/.

3. RESULTS

3.1. Prediction of H. pylori Virulence Interactome

In order to have general overview of genes involved in H. pylori pathogenesis and the prediction of H. pylori VRN, extensive literature survey was conducted and different protein regulators were predicted that are involved in global regulation of the pathogenic genes (factors) (Figure 4). These included Fur, ArsS-ArsR system, CsrA and NikR. Among the 17 transcriptional regulators of H. pylori (Danielli, et al., 2010) fur which codes for metallo-regulatory protein ferric uptake regulator 'Fur', was the first identified transcriptional regulator necessary for the growth and colonization of pathogen in gastric mucosa under acidic and iron limiting conditions. Additionally it also has influential control the expression of metabolic and energy production related genes (van Vliet, et al., 2003, van Vliet, et al., 2004). Protein Fur further expands the spectrum of its regulation by playing its dual role as iron complex form of Fur (Fe-Fur) as well as apo form of the enzyme (apo-Fur) (Ernst, et al., 2005). Fur (Fe-Fur and apo-Fur) regulates the related expression by binding to Fur box sequences located inside the promoters of metabolic genes. (Pich, et al., 2012). In, apo-form Fur regulates the expression of outer-membrane proteins (ompB and oipA), iron storage protein (pfr) and most important virulence factors (toxigenic genes); cagA and vacA while in Fe co-factored-form, Fur regulated the expression of flagellar genes (flbB and flaE) and genes implicated in iron homeostasis like frpB1,exbB2, fec1and fec2 (Ernst, et al., 2005). Free Fe is also capable of regulating the expression of several genes like vacA, cagA and other cag pathogenicity island genes (cag3, cag4 and cag26), outer-membrane proteins (hopA and omp6), fecA3 (involved in iron uptake) and transposition regulatory protein (tnpB) (Ernst, et al., 2005). ArsS-ArsR system like Fur also regulates the expression of genes involved in acid resistance (amiE and amiF; amidase and formamidase enzymes), urease operon, exbB2, dnaK operon (dnaK, hrcA and grpE; involved in heat shock responses), napA, omp6 as well as fur (Pflock, et al., 2006), sabA which in involved in adhesion to gastric cells(Goodwin, et al., 2008). ArsS-ArsR system can regulates its own expression as well (Pflock, et al., 2006). NikR is a metal responsive regulatory protein involve in nickel homeostasis (Contreras, et al., 2003). In the presence of excess nickel it upregulates the expression of nixA, a high-affinity nickel-transport protein (nixA represses the expression of urease operon) (Contreras, et al., 2003). While NikR represses the transcription of iron uptake and storage genes (pfr, fur, frpB4 and exbB/exbD), genes involved in motility (flaA and flab), genes implied in stress responses (hrcA-grpE-dnaK) and genes encoding outer-membrane proteins (omp6, omp11, omp31, omp32 and hopZ) (Contreras, et al., 2003). CsrA- carbon storage regulator, is a post-transcriptional regulator which functions as global regulator of genes involved in the acid induction of napA (gene encoding neutrophil activating protein), cagA, vacA, the urease operon (Important in colonization of gastric cells and acid resistance) and fur (Barnard, et al., 2004). It also regulates the heat shock responses of napA, groESL and hspR and the expression of ahpc (involved in anti-oxidant activity) (Barnard, et al., 2004). In addition to this, another transcriptional regulators HspR auto regulates its expression as well as regulates the expression of dnaK (Thompson, et al., 2003). Another transcriptional repressor HrcA auto regulates its expression in heat shock responses (Thompson, et al., 2003).

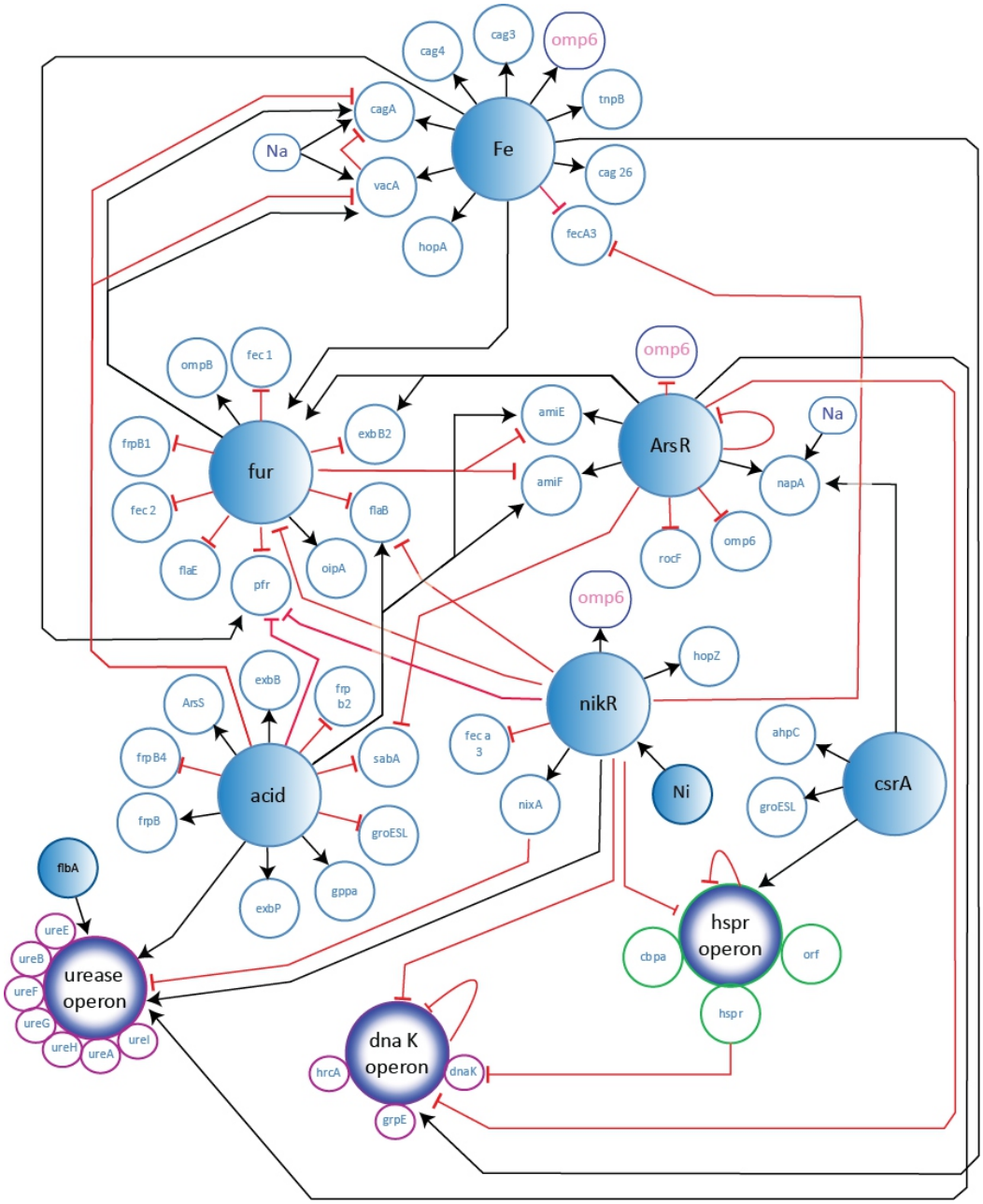

Figure 3. Derived extensive interactive network of virulence factors of H. pylori. The figure displays the detailed and complex interactions of various virulence factors of H. pylori .The network shows the differential expression of acid responsive genes, iron uptake genes, flagellar genes in acidic, iron depleted and salt conditions and their regulation by global regulators like Fur, csrA and two component systems like ArsR-ArsS system. All of the interactions were confirmed using reported laboratory findings however, the regulation of mentioned virulence factors except cagA and vacA were not confirmed through qualitative modeling. The objective of this study was to reveal the strategy that H. pylori employs to survive and persist in human gastric mucosa. There are number of components (gene and gene products) that are involved in the regulation of pathogen's virulence however, it is difficult and non-logical to include all of these components in a logical regulatory model. Therefore, such networks are constructed only to grasp the general overview of pathogenesis of bacteria. With this network in the background, only the most crucial virulence factors (cagA and vacA) of H. pylori were identified and included in the virulence regulatory graph, which was the modeled quantitatively. Such construction of a network structure was crucial for the prediction of virulence regulatory system of H. pylori. Due to complex and huge size of this network, intuition alone was not adequate to grab the dynamical behavior of H. pylori induced pathogenesis. Therefore, the next logical step was the prediction of specific virulence factors and their respective interactions. The interaction dynamic values assist in the generation of dynamical behavior of the targeted outcome of H. pylori induced pathogenesis. The straight arrow represents the activation while arrows with red dots show the inhibition of the subsequent genes.

The H. pylori colonizes highly acidic environment of stomach to adapt these harsh habitat the pathogen down regulates nine outer-membrane protein (OMP) related genes; hopD, hopA, homA, hopO, sabA, hofH, hopQ, horL, hopK and up regulates two genes horA and hp1467 (Mahdavi, et al., 2002). Acid activates ureA, ureB, ureF, ureG, ureH and ureI of urease operon. All these genes encode for urease enzyme as well as other genes involved in buffering of acidic environment are up-regulated. These genes include amiE and amiF encoding amidase and formamidase respectively (Merrell, et al., 2003). motB and hp1192, responsible for motility are also up-regulated in response to low pH (Bury-Mone, et al., 2004) e.g. genes encoding flagella rotation protein (Bury-Mone, et al., 2004) and flab which is involved in motility (Merrell, et al., 2003). On the other hand, acid (in stomach) also induce regulation of of genes involve in iron homeostasis (pfr, exbB, exbP, frpB, frpB2, frpB4), heat shock response (groESL, gppa and grpE), adhesion (sabA) and transcriptional regulators (fur and arsS component of ArsS-ArsR system (Merrell, et al., 2003)).

Interestingly, CagA and VacA are found in functional association. Protein CagA down regulates the vacuolating activity of VacA, similarly VacA reduces the activity of CagA. Studies have shown that CagA reduces the vacuolating and apoptotic effects of VacA, whereas, VacA reduces the formation of 'humming bird phenotype' induced by CagA. However, no genetic linkage is yet confirmed between vacA and cagA. Such association is reported only in host cell signal transduction. The antagonizing effect of both these entities prevents the gross damage to gastric epithelial tissues, helping the pathogen to modulate the secretion of its virulence factors and thus enable it to adapt to host cell environment (Argent, et al., 2008). The net VacA and CagA antagonizing effect on gastric epithelial tissue vary widely between strain types. Further insight into the genetic as well as molecular aspects associated with VacA and CagA signal transduction in host cell can provide better understanding of physiological interaction between the two important cytotoxins of H. pylori (Palframan, et al., 2012).

The GINsim tool is used for the qualitative (discrete) modeling of VRNs according to René Thomas formalism; it considers VRN as a directed graph along with logical parameters and in result generates the state graph, where stable states, cycles and acyclic paths between any states can be identified.

3.2. Logical Parameters (K) of cagA-vacA VRN

The logical parameters for the VRN were computed by the formula as discussed in methodology. These logical parameters were computed for the entities used to construct original cagA-vacA regulatory graph; these entities were cagA, vacA and Fur respectively (Figure 2). Table 1 represents logical parameters of each entity present in cagA-vacA VRN.

3.3. State Graph Generated from cagA-vacA VRN

The state graph was obtained after the optimization of logical parameters of biological regulatory network. The state graph encompasses all the feasible qualitative states or configurations. Each state portrays distinct expression of an entity at a particular moment of time. Moreover, each single state shown in a state graph is a combination of entities assigned in the original VRN. The cagA-vacA VRN shows each state as the qualitative expressions of cagA, vacA, Fur, Fe, acid respectively. The state graph of our VRN encloses 14 states including one stable state (Figure 3). These states were verified and explained in connection to the support from previous laboratory findings.

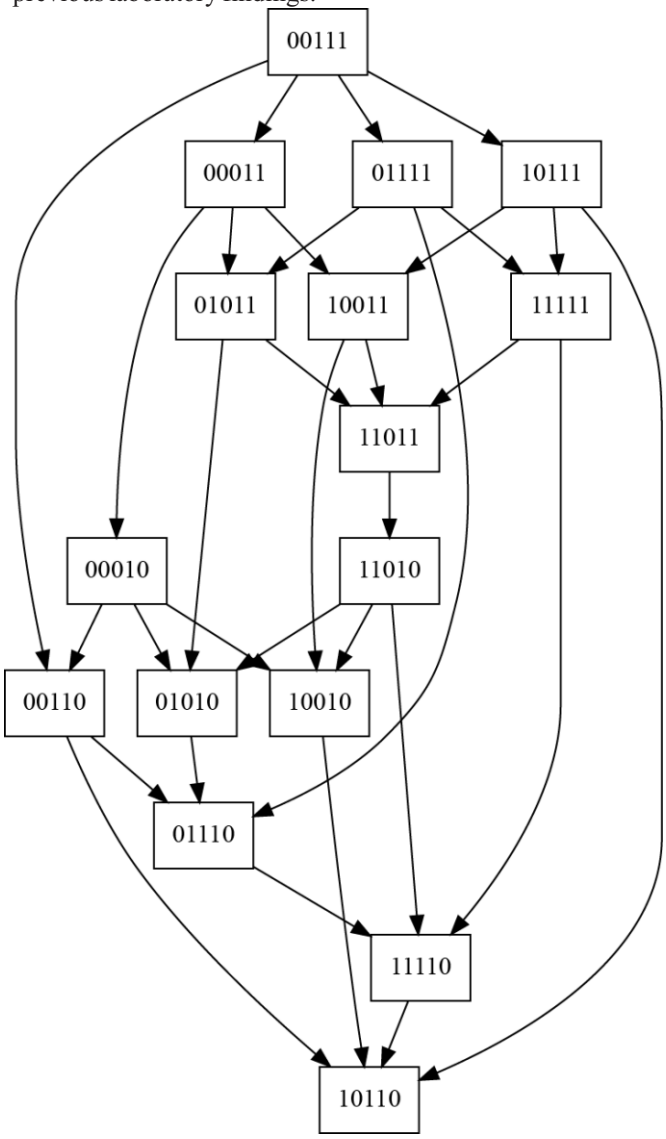

Figure 4. State transition graph of cagA-vacA VRN. State graph or of Virulence Regulatory Network helps in prediction of regulatory pathways that are further confirmed by reported laboratory findings. The cagA-vacA VRN depicts the regulatory pathways implicated in the achievement of stable state, gastric carcinogenesis through the combination of virulence factors cagA, vacA and regulatory protein Fur in acidic and iron depleted environment of gastric mucosa.

3.3.1. Analysis of the State Graph (States and their Transitions)

The starting state of the cagA-vacA VRN (state graph) is 00111. This state indicates the activation of Fur under iron limiting and low pH conditions it also indicates the condition when H. pylori adheres to the gastric epithelium of gastric mucosa (Raghwan & Chowdhury, 2014). After the adherence (00111), there are three separate states (00011, 01111 and 10111) emerging simultaneously and ultimately leading to the deadlock state (10110). The path involves the state transition from 00011 to 10110 i.e. mucosal damaged condition to the development of adenocarcinoma. For instance, the expression of cagA and vacA is immediately inducted separately in two of these states (01111 and 10111) after the adherence process. The state transition from 00111¬¬ to 01111 depicts the ongoing gastric colonization after adhering properly to gastric epithelium. Meanwhile vacA and fur are expressed simultaneously as both are extremely important factors for early successful colonization of gastric niche (Merrell, et al., 2003, Gancz, et al., 2006, Oldani, et al., 2009, Miles, et al., 2010, Pich, et al., 2012).

In an adhered state, gene vacA become activated in a Fe-Fur dependent manner while cagA merely by Fur (apo-Fur). It has been observed that absence or inactivation of Fur leads to impaired colonization conditions (Merrell, et al., 2003). Simultaneously the state transition 00111 to 10111 is crucial for pro-inflammatory responses after successful adherence. Gene cagA is induced in the host colonized epithelium which then promotes pro-inflammatory and anti-apoptotic effects since vacA (promotes apoptosis of the gastric epithelial cells) is shutdown in this state (Oldani, et al., 2009). The continued pro-inflammatory actions of state (10111) lead to the inflammation of gastric lining (11111) i.e gastritis. This inflammation is however not detrimental since vacA is switched-on at this state (11111) dilutes the disastrous effects of cagA induced inflammation and damage (Ruggiero, et al., 2006). This state will logically lead to more stable and long-lasting state (11011) clinically known as chronic gastritis (chronic inflammation of gastric mucosa also referred as chronic gastritis).

The chronic gastritis is pre-disposition to the atrophic gastritis, long-lasting ulcers that eventually results in cancer (Roesler, et al., 2011). Meanwhile, the state 01111 assists in building the state 01011, which ultimately leads to the state 11111. The state 01111 signifies the enhanced activation of vacA in the absence of its inhibitor cagA which rolls up in exaggerated vacuolating action of toxin and hence vacuolation of gastric epithelial cells. The increasing vacuolating toxicity (01111) results in extensive apoptosis of gastric cells which further advances to the immunomodulatory condition (Roesler, et al., 2011), atrophic gastritis (Israel & Peek, 2001) (11011) through early maintenance of the disease (10011) (Roesler, et al., 2011) and chronic gastritis (11011). The long-lasting and chronic conditions of gastritis (11011 and 11010) in conjunction with muted T-cell response acts as key precursors in the formation of premalignant lesions (Leung, et al., 2004) known as intestinal metaplasia (10010). The intestinal metaplasia (10010) through dysplasia finally falls into the stable state of gastric carcinogenesis (10110). No recent study suggests the clear correlation of gastric adenocarcinoma with other types of cancers therefore gastric adenocarcinoma has been assigned a deadlock or stable state of agA-vacA VRN as shown in Figure 5 (de Martel, et al., 2008, Koshiol, et al., 2012).

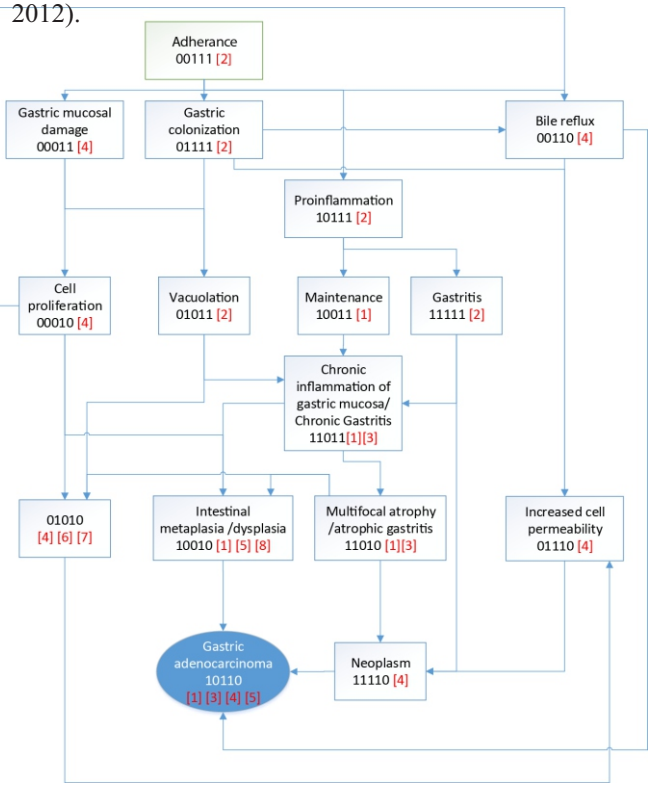

Figure 5. Schematic representation of the cagA-vacA state transition graph. The stable state of the graph is represented by state 10110, which is a potential EVENT of gastric carcinogenesis (Sobala, et al., 1993, Cahill, et al., 1994, Lynch, et al., 1995, Ricci, et al., 1996, Warburton, et al., 1998, Oldani, et al., 2009, Roesler, et al., 2011).

Adherence (00111) of H. pylori to gastric cells can cause gastric mucosal damage (00011). This results in the repression of cagA and vacA under low pH conditions which lead to expression of other gene which are not discussed here as they are not included in cagA-vacA VRN and are involved in epithelial damage. After gastric mucosal damage (00011), altered cell proliferation (Lynch, et al., 1995) is observed in gastric cells (00010). Gene cagA does not have any effect on cell proliferation while vacA specifically inhibits cell proliferation (01010). It has been proved in previous studies that increase in altered cell proliferation is not associated with the severity of gastritis rather it is the direct effect of the pathogen (Ricci, et al., 1996). Cell proliferation is now considered as one of the earliest changes in mucosal lining and acts as catalyst in the development of adenocarcinoma (Cahill, et al., 1994). Several studies have proved this development as abrupt increase in mucosal cell proliferation which leads to the accumulation of neoplastic clone of cells (11110) especially in the presence of bile reflux (00110) and chronic epithelial damage (00011) (Lynch, et al., 1995). Bile reflux (00110) also known as 'reflux' or chemical ' gastritis'

increases the cell permeability (01110). The presence of these agents (00110, 00011 and 01010) contributes synergistically to the development of intestinal metaplasia and is consistent with the laboratory findings. It is a well-known fact that intestinal metaplasia and dysplasia always proceed before the development of neoplasm, however, intestinal metaplasia/dysplasia may or may not proceed towards neoplasm. The neoplastic clone of cells along with other etiological agents (11111, 11010 and 01110) establishes the stable state of gastric adenocarcinoma /gastric carcinogenesis (Lynch, et al., 1995).

The model is usually confined to target a single behavior or outcome. The shortest possible route toward the normal sink was also identified and used as the reference trajectory (path) as shown in Figure 6. The small divergences from this trajectory that culminate at the stable state have been discussed.

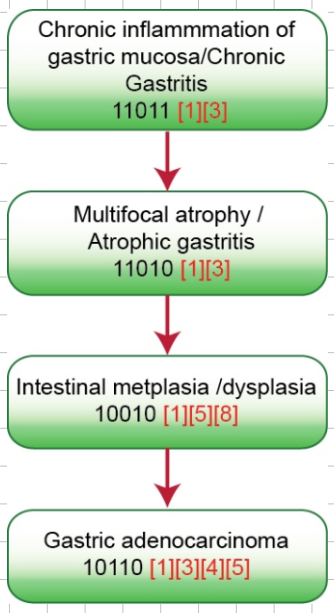

Figure 6. Shortest route to stable state. The shortest possible route to adenocarcinoma is derived, which is in agreement with the clinical findings, epidemiological data and prospective histo-pathological studies.

3.3.2. Analysis of the State Graph with Respect to pH and Iron Conditions

The inflammation is usually inducted for the provision of nutrients to H. pylori species thus Fe=1 in the following states; pro-inflammation (10111) leads to acute gastritis (11111) followed by chronic gastritis (11011) until it reaches atrophic gastritis (11010) because continued inflammation is not very favorable for the survival of the pathogen. Thus reduction of inflammatory conditions leads to the stable, immune-compromised and premalignant states of atrophic gastritis (11010) and intestinal metaplasia/dysplasia (10010) (Blaser, 1995).

Increased acid production is evident in inflammatory states (10111, 11111, and 11011) (Blaser, 1995, Israel & Peek, 2001, Leung, et al., 2004), while decreased acid production or hypochlorhydria (acid=0) are the characteristic of atrophic cells (atrophic gastritis/11010) and premalignant lesions (intestinal metaplasia/10010).

4. DISCUSSION

A cell, whether prokaryotic or eukaryotic, responds to external stimuli by the coordination of various biological processes. These biological pathways include interactions as well as regulation of gene, protein, signal transduction or metabolic process. For instance, at gene level, various transcriptional regulatory proteins regulate either self-expression or activity of other genes. The repression or activation of respective genes by these regulatory proteins leads to either increase or decrease in number of mRNA transcripts. This ultimately changes the concentration of respective proteins, which leads to new state or new behavior of the cell. All these genes and their products constitute a dense network of interactions in which one component interacts either positively or negatively with other components. In a miniature world of such myriad components and their intricate interactions in biological systems, network modeling assists in representing the entire system in cohesive outline (Karlebach & Shamir, 2008).

H. pylori - a persistent pathogen is able to resist and flourish in acidic environment of the stomach and under iron limiting conditions. Our proposed cagA-vacA VRN suggested the pathway, that leads to the gastric carcinogenesis, emerges by the combination of fluctuating expressions of cagA, vacA and Fur under low pH and iron depleted conditions. Nearly all individuals colonized by H. pylori have co-existing gastric inflammation; however, only a small percentage of colonized individuals follow the proposed sequela. Potential risks are due to the combinations of variable expression of bacterial gene products to differences in the intensity and magnitude of host inflammatory response to pathogen or distinct interactions between host and the pathogen (Israel & Peek, 2001).

The pathogen after adhering successfully to the host cells can provoke a series of inflammatory conditions causing gastric mucosal damage and gastric colonization. Unlike other bacteria (e.g. Mycobacterium tuberculosis) which inhibit its host for many years but remain mainly in dormant or latent form, H. pylori cause persistent and continuous inflammation (Cadamuro, et al., 2014). Such chronic and long-lasting conditions of inflammation can lead to the development of atrophic gastritis and formation of pre-malignant lesions such as intestinal metaplasia, dysplasia and neoplasm, which altogether create the fate of gastric carcinogenesis.

The Fur has been found to play critical role in productive gastric colonization; however, it is certainly not required for persistent infection (Gancz, et al., 2006). Therefore, the events following adherence and gastric colonization, does not require fur expression. VacA is another important factor in successful colonization of gastric mucosa. Colonization of the pathogen initiates strong systemic immune response which promotes the chronic inflamed environment of gastric mucosa, chronic gastric inflammation or chronic gastritis through enhanced expression of cagA for its preparation in pro-inflammatory effects (Israel & Peek, 2001). The early maintenance of disease and co-expression of cagA and vacA

in persistent gastritis drives gastric carcinogenesis. The vacA, in absence of interference from cagA, promotes vacuolation and subsequent apoptosis of the gastric cells. The cagA expression along with vacA counteracts the damaging effects of vacA, which is harmful for host and also for the survival of H. pylori The co-expression of both toxigenic genes also assists establishing the state of chronic inflammation, which is a risk factor for the gastric malignancy. According to few studies, the acid production increases in a state of chronic inflammation, most likely results from the increase in serum gastrin acid and decreased somatostatin levels caused by gastric inflammation/gastritis (Israel & Peek, 2001).

In a state of chronic inflammation, there is a remarkable increase in cell apoptosis. The ability of H. pylori to alter the apoptosis influences its associated clinical outcomes of disease. The enhanced apoptosis accelerate the process of cell demise and hence contribute to the atrophic gastritis with increase in the risk of gastric carcinoma. In contradiction, the reduced rates of apoptosis especially in the presence of hyper proliferation catalyze the retention of mutagenized cells which in turn make H. pylori infected individuals liable to the gastric cancer. The atrophic gastritis is often accompanied by intestinal metaplasia (Ohkuma, et al., 2000, Israel & Peek, 2001).

The H. pylori induced injury by inflammatory cells not only inducts DNA damage but also promotes the production of radicals that are responsible for accumulation of mutations and malignancies. The pathogen thus provides a breeding ground for the formation of pre-neoplastic and neoplastic lesions through acute and chronic gastritis (Guarner, et al., 1993). Cell proliferation induced as a direct effect of pathogen is regarded as the pioneer inducer of mucosal changes in the development of gastric adenocarcinoma. In the normal mucosal environment, the undifferentiated cells undergo active cell proliferation in the gastric pits and neck region of the glands. These newly formed cells after maturation and differentiation migrate to the surface of mucosa and arrange themselves in columnar cell layers at lumen. Studies have confirmed that the highest rate of cell proliferation is at the base of gastric pits in healthy mucosa. However in H. pylori infected mucosa, particularly in case of H. pylori associated atrophic gastritis, there was enhanced cell proliferation throughout the entire gastric pits (Cahill, et al., 1994). The pathogen also inhibits the secretion of ascorbic acid entering the stomach thus lowers the gastric juices ascorbic acid levels. It has also been proved that eradication therapy can restore the normal ascorbic levels in gastric juices. Bile reflux has been proved to play supporting role in the H. pylori induced gastric adenocarcinoma due to its carcinogenic properties; however, there is no recorded data available that proves the origin of bile reflux due to H. pylori infection. Several studies have been conducted to confirm the link between gastric colonization and bile reflux in patients that were undergone through gastric surgery. The results have shown that postoperative 'chemical gastritis' play an important role in the eradication of H. pylori. Furthermore, it was proved that H. pylori again colonize (Ladas, et al., 1996) the gastric mucosa after bile diversion surgery. Despite of all the above observations, H. pylori and bile reflux co-exist as the bile acid samples collected from patients that are not undergone through gastric recession are much lower in concentration than collected from post-operative gastric samples; this proves that H. pylori species are able to withstand the harmful effects of bile reflux in intact stomach.

Bile reflux also promotes the enhanced cell permeability, which in turn expose the nuclei of gastric epithelial to the mutagens present in lumen. This leads to the mutagenesis preparing for the final stage of H. pylori related disease outcomes i.e. gastric carcinogenesis (Lynch, et al., 1995). The carcinogenic nature of bile reflux along with chronic epithelial damage and ever-increasing cell proliferation leads to the accumulation of mutations, under pathological and physiological stress the mature gastric tissues start to decrease in number and immature cells begin to increase. Two independent studies have shown that the increasing risk of gastric cancer depend upon these two pathways; 1) vacA expression in gastric epithelial cells causing vacuolation and subsequent apoptosis and 2) the increasing cell proliferation in intestinal metaplasia and dysplasia supported by cagA expression. Increasing cell proliferation that is not balanced by the synergic increase in apoptotic indices over years of colonization support the carcinogenic ability of cagA+ strains towards increasing risk for gastric carcinogenesis (Israel & Peek, 2001). The ongoing combination of stimuli leads to the buildup of neoplastic cell clones that eventually become malignant and drive the pathway to gastric carcinogenesis.

5. CONCLUSION

The pathogenic nature of H. pylori and its adaption to the stressful environment of gastric mucosa is attributed to the regulation and differential expression of virulence factors by few specific transcriptional regulators. Our proposed study highlighted the differential expression of Fur and its influence on the expression of cagA and vacA in addition to low pH and iron levels. All of the above-mentioned conditions assist in constructing various regulatory pathways that help to attain the ultimate disease outcome, gastric carcinogenesis. The high-grade inflammation is inducted by cagA under hyperactive secretion of acid while vacuolation, apoptosis and atrophy mediated by vacA under hypo secretion of acid. The expression of both genes helps to regulate and modulate the damaging consequences in the formation of long lasting disease conditions like chronic gastritis and atrophic gastritis. These conditions assist the formation of pre-malignant lesions through increased cell permeability and in combination with aberrant cellular proliferation that ultimately contributes in the developmental of carcinogenesis. The proposed cagA-vacA VRN has presented in simplified form to understand the pathogenesis and regulation of two virulence factors by global regulator Fur under low pH and limited availability of iron. However, many other virulence factors may influence the regulatory pathway of gastric pathogenesis like ureA, ureB, ureF- ureI, napA, ferric iron transport genes including frpB1, frpB2, feoB etc. Moreover, the genes implicated in acid acclimation and iron deficiency is regulated by other regulators in addition to Fur. The proposed cagA-vacA VRN may not applied be to all H. pylori strains and hence cannot be considered as thumb rule for the ultimate pathogenic nature of H. pylori.

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