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Original research article

Expression of *Helicobacter pylori* Virulence Genes under Atmospheric Condition

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ABSTRACT

Helicobacter pylori, a dimorphic gram-negative microaerophilic bacterium, is a major causative factor for many gastrointestinal diseases and is also a carcinogenic agent of gastric cancer. *H. pylori* is capable of surviving in adverse environments by transforming its shape from spiral to coccoid. It was previously reported that the atmospheric oxygen-induced coccoid *H. pylori* retained its ability to produce virulence factors. In this study, the coccoid mRNA levels of the major virulence genes were analyzed by quantitative RT-PCR using the SYBR green fluorescein method. *H. pylori* was converted into its coccoid form by incubating it in an atmospheric oxygen environment; the expression of virulence genes while in the coccoid form were compared with that of the bacteria in spiral form. After 24 hours of oxygen exposure, approximately 85.52% of the bacteria were coccoid-shaped. Interestingly, mRNA for all 10 virulence factors studied herein were continuously produced, with the highest up-regulation occurring for the *cagA* gene, increasing by over 2.5-fold ($P < 0.01$). The information in this study provides insight into the functions of coccoid *H. pylori* and how it may still remain infectious in adverse environments.

Keywords: *Helicobacter pylori*; Virulence gene; Atmospheric oxygen

1. Introduction

Helicobacter pylori, a gram-negative microaerophilic bacterium, is a major causative factor for many gastrointestinal diseases including chronic gastritis, peptic ulcers and gastric cancer [1]. *H. pylori* infections in humans is reported to occur for over 50% of the global population [2]. The bacteria can be found in two different forms, the typical spiral shape, and the viable but non-culturable (VBNC) coccoid form [3]. It has been noted that the bacteria can transform its shape from spiral to coccoid when encountering unsuitable environments such as those that have a high oxygen concentration, anaerobic conditions, prolonged cultivation, or are undergoing antibacterial treatment [4-11]. The coccoid *H. pylori* has also been found in the human stomach where the environment is extremely antagonistic to bacterial existence [12]. It was reported that the coccoid bacteria are in

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fact alive, as their abiliy to generate ATP and express some virulence genes was observed [4]. However, they cannot be grown on culture media by regular techniques. In addition, it is important to mention that the coccoid *H. pylori* are still infectious because their ability to produce some major virulence factors remains in place. The virulence and pathogenicity of coccoid *H. pylori* have been reviewed in detail elsewhere [3-6, 13-14].

H. pylori virulence factors are necessary for the bacteria's survivability and pathogenicity in the human stomach. These factors are classified according to their functions as exotoxin/ endotoxin groups, colonization factors/colonization associated enzymes, auxiliary factors, inflammatory stimulating factors, immune system evasion factors and bacterial defense factors [15]. The virulence factor CagA protein, encoded by the cytotoxin-associated gene A (*cagA* gene), stimulates the production of interleukin-8 which is a part of the type IV secretion system. The VacA protein, a product of the vacuolating cytotoxin gene A (*vacA* gene), induces vacuolation in eukaryotic cells. The *ureA* and *ureB* genes encode the UreA and UreB subunits of urease which plays an important role in acid neutralization in the host stomach. The γ-glutamyl transpeptidase (GGT), the product of the *ggt* gene, influxes glutamine from host cells into the bacterial cytosol and produces ammonia for acid neutralization in combination with urease. The *rocF* gene encodes arginase (RocF) that facilitates production of ammonia and NO in stimulated macrophages. The blood group antigen binding adhesin (BabA), encoded by the *babA* gene, binds to fucosylated Le^b blood group antigens on host cells and is involved in inflammation, while the *sabA* gene product known as sialic acid-binding adhesin (SabA) binds to sialyl-Le^x and sialyl-Le^a antigens and is involved in activation of neutrophils. The outer inflammatory protein or OipA, encoded by the *oipA* gene, assists in IL-8 induction. The *napA* gene encodes the *H. pylori* neutrophil-activating protein (HP-NAP) which activates neutrophils and possibly binds to mucin.

Coccoid *H. pylori* are still infectious because they retain the ability to produce some major virulence factors [3-6]. Maneethorn et al. [16] demonstrated that the atmospheric oxygen-induced coccoid bacteria still produced γ-glutamyl transpeptidase, a virulence factor involving bacterial colonization. To date, information on virulence gene expression in coccoid *H. pylori* is limited and remains unknown for a number of adverse conditions. It was found that the decrease in *H. pylori* size, i.e., coccoid form, due to unfavorable conditons was correlated to the existence of some virulence genes [17]. To the extent of our knowledge, transcriptional levels of *cagA*, *vacA*, *ureA*, *ureB*, *rocF*, *babA*, *sabA*, *napA*, and *oipA* genes have not been studied in oxygen-stimulated coccoid *H. pylori*. These genes were chosen based on the roles of their gene products: acid escape virulence factors (UreA, UreB, RocF), epithelial cell colonizing factors (BabA, SabA, OipA, NapA), and epithelial cell pathogenicity factors (CagA, VacA, GGT). In this study, the mRNA levels of coccoid virulence genes, which are associated with bacterial colonization in the host stomach and development of gastrointestinal disorders, were investigated by quantitative RT-PCR with the SYBR green fluorescein method. The knowledge obtained should provide further insight into coccoid *H. pylori* infections and pathogenesis. In addition, this information will give an awareness to the risk of coccoid *H. pylori* that may be present in ambient environments and still remain infectious.

2. Materials and Methods

2.1 Bacterial strain and culture conditions

H. pylori test and control groups (reference strain ATCC® 43504™: urease positive, oxidase positive, and catalase positive) were grown on brain-heart infusion

agar (BHI; Difco, MD, USA) containing 7% blood at 37°C in microaerophilic conditions (5% O_2; 10% CO_2; 85% N_2) for 48 hours in an anaerobic jar with an AnaeroPack®-MicroAero gas generating system (Mitsubishi Gas Chemical Company, Inc, Tokyo, Japan).

For the test group, *H. pylori* were transferred from a microaerophilic environment in the anaerobic jar to the atmospheric environment (21% O_2; 0.1% CO_2; 78% N_2) at 37°C for 6 and 24 hours. For the control group, bacteria were further grown in microaerophilic conditions for 6 and 24 hours. The morphology of *H. pylori* at different periods and conditions were observed, as detailed below. At the same time, *H. pylori* cells from the same bacterial culture were collected, resuspended in BHI broth, and centrifuged at 6,000 rpm for 5 minutes. Supernatant was discarded, and the cell pellet was collected and kept at -80°C until later use [18].

2.2 Bacterial morphology

Bacterial cells were smeared on a slide and stained by Gram's method. The bacterial morphology was observed under a light microscope using 100x oil immersion objective lens, as is described elsewhere but with a small modification [19]. The number of bacterial cells (both spiral- and coccoid-shaped) were counted for at least 3 high power fields (approximately 500 bacterial cells) and % coccoid-shaped bacteria was calculated per total bacterial cells.

2.3 RNA isolation and cDNA synthesis

Total RNA was isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA) as previously described by Chomczynski and Sacchi [20], then subsequently converted to cDNA and used as the PCR template for a transcriptional study of the *H. pylori* virulence genes. RNA samples were tested for their concentration, purity, and quality. The purity and concentration of the RNA were verified spectrophotometrically at 260 and 280 nm [18]. An A_{260}/A_{280} ratio between 1.8 to 2.0 indicated an acceptable level of purity for the RNA. The quality of the RNA was verified by 1% agarose gel electrophoresis in TBE buffer (0.089 M Tris, 0.089 M borate, and 0.002 M EDTA). The pattern of the ethidium bromide-stained RNA was visualized by a gel documentation system under UV light.

Prior to converting the RNA to cDNA, residual DNA was eliminated from the RNA samples by DNaseI (Thermo scientific, MA, USA) and then was rechecked by PCR, using the treated RNA sample as the PCR template. For DNaseI treatment to be considered successful, no amplified DNA was to be observed after the DNA amplification process.

2.4 Primer design and primer efficiency

Primers for the *H. pylori ureA*, *ggt*, *babA*, *sabA*, *napA*, and *16S rRNA* genes were synthesized according to the published sequences [21-24] whereas those for the *cagA*, *vacA*, *ureB*, *rocF*, and *oipA* genes were designed in this study using the Primer3 program (http://bioinfo.ut.ee/primer3/) as shown in Table 1. All primers were checked for their amplification efficiency before performing the qRT-PCR; this was carried out through a set of 10-fold serial dilutions of the cDNA, followed by qPCR. The C_T of the amplification (Y-axis) was plotted against the Log of cDNA template concentration (X-axis) and the primer efficiency was calculated as follows:

$$\text{Primer efficiency (\%)} = (10^{(-1/\text{slope})}-1) \times 100$$

Table 1. Primers for *H. pylori* virulence and housekeeping genes.

Gene	Primer sequence (5' to 3')		Product length (bp)	References
	Forward	Reverse		
cagA	AGCAAAAAGCGACCTTGAAA	AGCCAATTGCTCCTTTGAGA	172	This study
vacA	CAAAGCGCGGGACTAAACAT	CAATGACTTGCGTGGGTTGA	196	This study
ureA	GAAGACATCACTATCAACGAAGGCAA	GTTACCGCCAATGTCAATCAA	231	21
ureB	CGGCTGAAGACACTTTGCAT	CGATCGCTGGGTTAATGGTG	213	This study
ggt	CAGCCAGATACGGTTACGC	CCACCTGTCCGCTACAGAAT	99	22
rocF	ACCGAACAGAGCGAAAGAGA	GCTCATCAAAACTCAGCCCG	217	This study
babA	GCACCCTAAACACCCTTATCAAA	ATACCCTGGCTCGTTGTTGAA	254	23
sabA	AGCATTCAAAACGCCAACAA	AAAAACCCAATACCGAAGTGATAA	145	23
napA	GCGATCAAACTCACTCGTGTTAAA	AATGGATTTTTGCAACTTGGCTAA	195	23
oipA	CTCTCTCGTTCTGGCTCCAC	AGGCGTTTTGTGCATCTCTT	195	This study
16S rRNA	GCTCTTTACGCCCAGTGATTC	GCGTGGAGGATGAAGGTTTT	172	24

2.5 Virulence gene expression analysis by Quantitative Reverse Transcription-PCR (qRT-PCR)

The cDNA was used as a template for the qRT-PCR. The reverse transcription was performed by the Superscript™ III First-stand Synthesis System (Invitrogen, USA). The gene expression levels were analyzed by the qPCR with SYBR Green dye using an Applied Biosystems StepOnePlus™ PCR machine. To quantify the mRNA level, 100 ng of the cDNA was added into a well of an optical 96-well reaction plate containing a mixture of 200 nM primers and 1x QPCR green mastermix HROX (Biotechrabbit, Berlin, Germany). The plate was covered with an optical sealing film prior to placing it in the qPCR machine. The qPCR process consisted of an initial denaturation step at 95°C for 2 minutes, followed by 40 amplification cycles of a denaturation step at 95°C for 15 seconds, an annealing / extension step at 60°C for 30 seconds, and a final step for melting curve analysis at 72°C for 5 minutes. The *16S rRNA* gene was used as an endogenous control and water was used as a negative control. The experiment was done in triplicate. The relative quantification of the gene expression was expressed as fold change, which was determined by the $2^{-\Delta\Delta C_T}$ method [25].

2.6 Statistic analysis

Statistical difference between groups was compared by Student's T-test using SPSS for Windows. A *P*-value of less than 0.05 was accepted as indicating a significant difference.

3. Results and Discussion

3.1 Induction of the coccoid-shaped *H. pylori* by atmospheric oxygen

Spiral-shaped *H. pylori* were transformed into the coccoid shape through oxygen exposure for various incubation periods (Fig. 1). At 6 and 24 hours of oxygen exposure, approximately 28.57% and 85.52% of the spiral-shaped bacteria were transformed into the coccoid shape, respectively. It appeared that the longer the bacteria were exposed to oxygen, the higher the proportion of coccoid bacteria rose. This data is in concordance with the observation of Cellini *et al.* [7]. Since not all spiral bacteria were changed to coccoid after 24-hours of O_2 exposure, the expression of the remaining spiral bacteria could be taken into account.

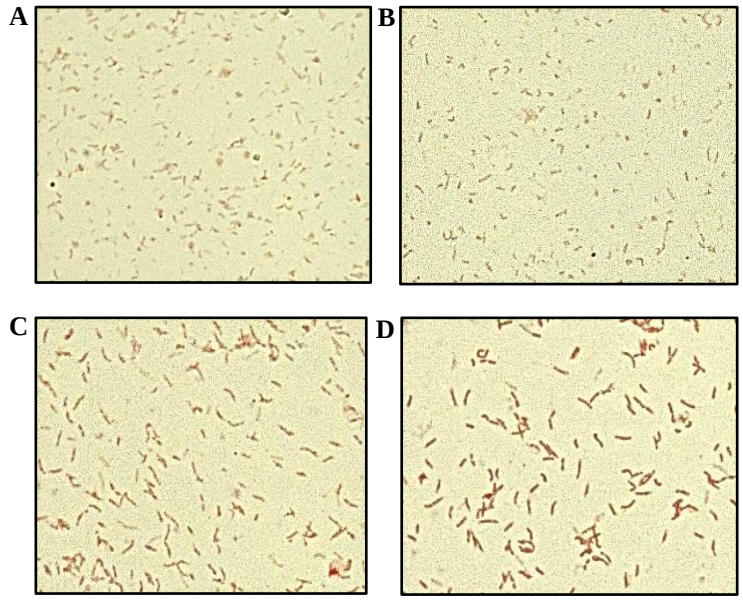

Fig. 1. Transformation of the spiral-shaped *H. pylori* into the coccoid form in atmospheric and microaerophilic conditions at different incubation periods. The picture was investigated by 100x magnification under light microscope.
(A) 6 hours in atmospheric conditions (28.57% coccoid),
(B) 24 hours in atmospheric conditions (85.52% coccoid),
(C) 6 hours in microaerophilic conditions (12.84% coccoid),
(D) 24 hours in microaerophilic conditions (24.93% coccoid).

It is noteworthy that the coccoid conversion rate at 24 hours for the microaerophilic culture was 1.94 times what it was at 6 hours. Theoretically, the number of coccoid at these two intervals should not be much different. This finding may be due to the short external air influx that came while opening the anaerobic chamber to take the test group out to an atmospheric environment and to change the gas pack to keep the environment inside the chamber microaerophilic for the control group. As is known, an atmospheric oxygen amount (21%) affects bacterial survival. Oxygen is a precursor of free radicals and *H. pylori*, being a microaerophile, has fewer enzymes for removing these free radicals compared to other organisms [26]. Therefore, the bacteria protect themselves from free radicals by changing their shape into one which can better tolerate such unfavorable conditions [10]. The mechanism of coccoid induction is still unclear. It was proposed that environmental stresses e.g., long-term culturing, aerobic exposure (as in this study), and anaerobic exposure induce the expression of certain regulatory systems for lipid metabolism in *H. pylori* resulting in lipid composition alteration. This modification plays an important role in inducing the coccoid transformation [27].

3.2 Virulence gene transcription of oxygen-induced coccoid *H. pylori*

PCR efficiency of the virulence gene specific primers were determined prior to the qRT-PCR process. The results revealed that all of them were within the acceptable range (90-110%) indicating that the number of copied DNA molecules could double during each amplification cycle.

The levels of virulence gene expression were shown by the normalized threshold cycle (ΔC_T) of the target genes

(using *16S rRNA* gene as the reference) as indicated above each vertical bar in Fig. 2. The expression of the virulence genes of *H. pylori* cultured in atmospheric oxygen (Fig. 2A) showed that all genes looked at in this study were expressed during the 6 to 24 hours of culturing.

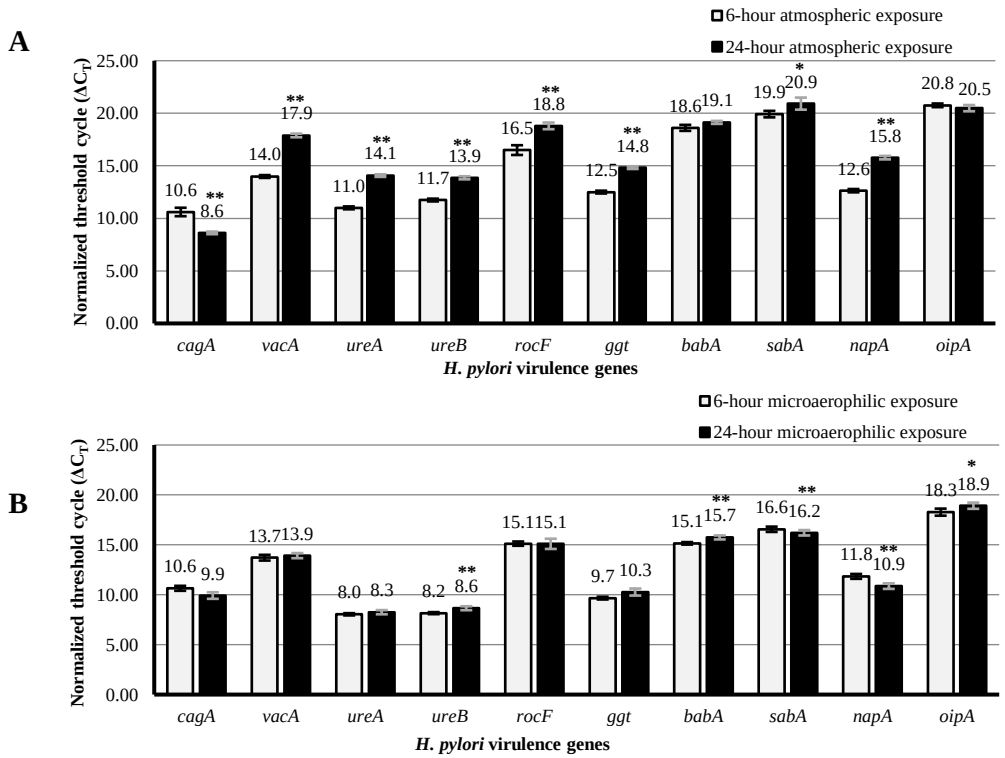

Fig. 2. Normalized threshold cycle (ΔC_T) of *H. pylori* virulence gene expression under atmospheric (A) and microaerophilic conditions (B). Data is presented as mean ± standard deviation of triplicate samples. *$p < 0.05$, **$p < 0.01$.

It was also found that when the culture time was extended from 6 to 24 hours in atmospheric oxygen, expression of the *cagA* and *oipA* genes increased while those of the other genes decreased. In the same way, all of the virulence genes of *H. pylori* cultured under microaerophilic conditions were expressed during the period of 6 to 24 hours (Fig. 2B). During this time of microaerophilic culturing, the expression levels of *cagA, sabA*, and *napA* genes increased while those of the other genes decreased.

The relative gene expression of the target gene was calculated using the normalized C_T of the test (*H. pylori* cultured in atmospheric oxygen) and the control (those cultured in the microaerophilic environment). It was found that at 6 hours in the atmospheric oxygen environment, the relative expression of the *cagA* gene showed a 1.03-fold increase but the other genes showed a lower relative expression than those of the bacteria grown in micro-aerophilic conditions as shown in Fig. 3.

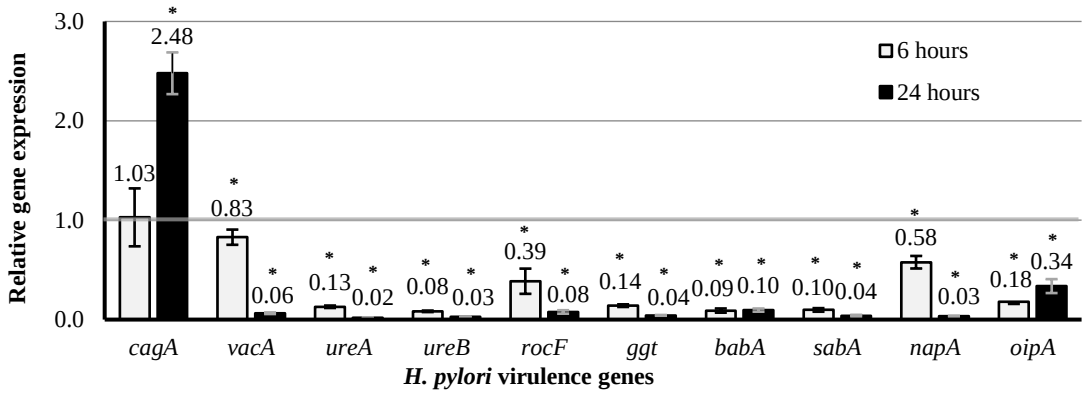

Fig. 3. The relative expression of the *H. pylori* virulence genes in the atmospheric environment (6 and 24 hours). Data is presented as mean ± standard deviation of triplicate samples. *$p < 0.01$.

In contrast, the relative expression of the *vacA*, *rocF*, and *napA* genes showed a slight decrease (0.83, 0.39, and 0.58 fold, respectively), while those of the *ureA*, *ureB*, *ggt*, *babA*, *sabA*, and *oipA* genes significantly decreased (0.13, 0.08, 0.14, 0.09, 0.10, and 0.18 fold, respectively) (Fig. 3).

At the 24-hour culture period in atmospheric oxygen conditions, the relative expression of the *cagA* gene significantly increased by a factor of 2.48, while the other genes showed a decrease over the 6-hour incubation period. The relative expression of the *vacA*, *urea*, *ureB*, *rocF*, *ggt*, *babA*, *sabA*, and *napA* genes were 0.06, 0.02, 0.03, 0.08, 0.04, 0.10, 0.04, and 0.03 times, respectively, with the exception of the relative expression of *oipA* gene that slightly increased (0.18 times at 6 hours, 0.34 times at 24 hours). This indicated that, during transformation, most bacterial virulence gene expression levels were reduced while the *cagA* gene expression level was increased.

The expression level of the *cagA* gene of *H. pylori* living in other adverse environments was previously reported elsewhere. Shao et al. [24] found an increased expression of the *cagA* gene when the bacteria were grown in the absence of serum. Monstein and Jonasson [14] showed that the *cagA* gene of *H. pylori* was continuously expressed after prolonged growth. It is likely that CagA is one of the crucial virulence factors for the coccoid *H. pylori* to be able to persist in unsuitable conditions. The latter investigators also reported that the expression of the coccoid *vacA* gene decreased after a long period of growth. This observation is supported by our findings in this study. As far as it is known, the virulence factors involved in *H. pylori* colonization are necessary for its survival. Although the surrounding pH of the bacteria appeared to be acidic as a result of metabolic waste accumulation, expression of the virulence genes associated with ammonia production, i.e. *ureA* and *ureB* (urease), *rocF* (arginase), and *ggt* (γ-glutamyl transpeptidase) were decreased ($p < 0.01$). The BabA, SabA, OipA, and NapA proteins play roles in host adhesion and inflammation. The decrease in these genes' expression during coccoid adaptation implies the impeding effect of O_2 exposure on bacterial colonization and/or infection [3]. The response to O_2 concerning NapA expression in this study was in agreement with the observation of Olczak et al. [28] in that not only NapA but also some other proteins take part in *H. pylori* oxidative stress protection. The mechanism of oxygen-induced coccoid transformation and its effect on virulence gene expression remain unclear. It was observed that the conversion from spiral to

coccoid form was associated with oxidative modification of both DNA and cellular proteins [29]. The association of virulence gene expression in coccoid *H. pylori* to the aspect of pathogenicity and treatment was not conclusively reported. It was noted that the conversion of the spiral-shaped bacteria to the coccoid form is associated with the bacteria's ability to escape the host immune response and the failure of antibacterial drug treatment [30].

Major metabolic changes occur during the VBNC state of bacteria; for example, there is a reduction in nutrient transport, respiration rate, and macromolecular synthesis [4] as well as gene expression, as shown in this study. With the exception of the *cagA* gene, the decreased expression of most virulence genes in this study was in agreement with previous reports in that the reduced transcription of some virulence genes during the conversion of the spiral *H. pylori* into the coccoid form was demonstrated [14, 16, 31].

4. Conclusion

A similar situation to the one observed in this study can occur when *H. pylori* contaminates the open environment where the amount of oxygen is toxic to the bacterium. In this unfavorable situation, *H. pylori* can adapt itself by taking on its coccoid form, which is viable but non-culturable. Notably, this form of *H. pylori* is transmissible and infectious since it still produces some virulence factors.

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