Determination of *ureA* and *hopQ* gene expression of *Helicobacter pylori* strains under acid stress conditions

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Abstract

Background: Acid pH induces transcriptional changes that affect the expression of *Helicobacter pylori (H. pylori) ureA* and *hopQ* genes, which may be adaptive mechanisms to acid stress conditions that are expressed differently depending on the virulence of the bacterium. However, the relationship between gene expression and virulence profile requires further studies, so we evaluated the effect of different pH values on the expression of *ureA* and *hopQ* genes of *H. pylori* strains with high and low virulence profiles.

Methods: Two *H. pylori* isolates with high virulence and low virulence profiles that were exposed to pH 3.0, 4.0, 6.0 and 7.0 for one hour under microaerophilic conditions were used. Subsequently, RNA was extracted from the isolates and cDNA was obtained to identify the differential expression of the *H. pylori ureA* and *hopQ* genes by semiquantitative real-time PCR. The results obtained did not present a normal distribution due to the small number of samples used; therefore, nonparametric tests such as the Mann-Whitney U test were used.

Results: This study revealed that *UreA* gene expression is higher in strains with a high virulence profile while *hopQ* gene expression increases in strains with a low virulence profile when subjected to acid stress. This result suggests that depending on the type of

virulence, *H. pylori* uses different mechanisms to colonize and adapt to the gastric environment and its pH fluctuations.

Conclusion: The results showed that in these two isolates with different virulence profiles there is an important effect of pH on the regulation of the expression of the *ureA* and *hopQ* genes. This possibly indicates that the change in pH will condition the adaptation mechanisms of each strain or isolate to the conditions of the gastric environment

Keywords: *Helicobacter pylori*, genetic expression, virulence factors, pH.

Introduction

H. pylori is a microaerophilic gram-negative bacterium that colonizes the surface of the gastric mucosa and duodenum. Moreover, *H. pylori* is associated with gastric and duodenale ulcers, chronic gastritis, gastric cancer, and Mucosa Associated-Lymphoid Tissue (MALT) lymphoma. There is evidence of relationship between this bacterium and some extraintestinal diseases such as iron and B12 deficiency anemias, idiopathic thrombocytopenia purpura, diabetes mellitus, cardiovascular diseases, and recurrent aphthous stomatitis (1).

H. pylori infection initially produces an immune response and superficial gastritis; however, no more than 10% of infected individuals develop symptomatic disease. The rest of infected population may remain asymptomatic or develop peptic ulcer or atrophic gastritis after years or even decades. The manifestation or lack thereof in patients may be due to genetic characteristics of the individuals, the environment, dietary factors, or *H. pylori* virulence factors (2–5).

The evolution of *H. pylori* infections is partly due to the expression of virulence factors such as cagA (cytotoxin-associated gene A), vacA (vacuolizing cytotoxin A) proteins, which change between strains, and the urease system. This explains the existence of *H. pylori* strains that are more virulent than others. cagA upon entering the host cell, activates kinases through EPIYA motifs at its C-terminus, which disrupt signaling pathways, increase interleukin-8 production, gastric inflammation and the risk of gastric carcinoma (2-5). Worldwide, cagA-positive strains are responsible for ~ 60% of *H. pylori* infections, compared with cagA-negative strains. Their association with carcinoma is due to their ability to promote proteasomal degradation of the TP53 protein and the acquisition of spontaneous loss-of-function mutations in the TP53 gene (6).

Meanwhile, *VacA* gene which includes two widely studied variable regions; namely, the signal region with two alleles, s1 (subtypes s1a, s1b, and s1c) and s2, and the middle region, with alleles m1 and m2 encodes a vacuolating protein that induces formation of cytoplasmic vacuoles inside epithelial cells, where it accumulates in different cellular compartments and generates apoptosis and acts as a urea transporter (5). This transporter can increase the permeability of the gastric epithelium to urea by enhancing urease activity. Furthermore, both *vacA* s1 and m1 genotypes are associated with increased neutrophil and lymphocytic infiltration, epithelial damage, gastric atrophy, and intestinal metaplasia (2–5).

At the same time, in order for this bacterium to colonize, grow and adapt to the acidic environment of the stomach, it modifies the expression of some genes related to the urease enzyme. This enzyme hydrolyzes gastric urea to form ammonia and neutralizes gastric acid, which allows *H. pylori* to invade the gastric mucosa and damage the epithelial cells (4).

Urease also stimulates inflammatory cells, producing further cell injury (5). This is demonstrated in a study published in 2018 by Marcus *et al*., which concluded that the *H. pylori* genes expression in the presence or absence of urea can vary when the acidity of the medium increases (7). Similarly, at least four microarray studies showed the existence of significant differences in *H. pylori* gene expression at neutral pH compared to gene expression when the bacteria are exposed to acidic pH (8–11).

In vitro studies show that exposing the bacteria to acidic stress may trigger the transcriptional response. This can produce variations in the gene expression like *cagA, vacA, UreA*, and *hopQ* due to changes in the microenvironment, where infections take place (7,12). Specifically, these studies showed that low pH induces the expression of the *cagA* gene. Meanwhile, the findings of *Merrell et al.* and *Allan et al*. suggest that *cagA* and *vacA* genes are strongly repressed when the bacteria is exposed to an acidic environment (10,13). However, exposing the bacteria to pH stress favors the expression of the *ureA* encoded by the urease gene cluster and involved in urea transport, as it rapidly increases ammonia production in the extracellular medium when the pH of the medium decreases (12). Additionally, *H. pylori* expresses about 64 Outer Membrane Proteins (OMPs), that facilitate adherence to gastric epithelial cells and are organized into at least five paralogous gene families. Family 1 is composed of the *Hop* and *Hor* genes. These genes encode for adhesion proteins and include hopQ, which is a gene that encodes for a protein that induces host cell signaling allowing translocation of cagA into the cells and decreases its expression in the presence of acidic pH (7,14).

As *H. pylori* passes through the stomach lumen, pH can change due to processes of cellular homeostasis and nutrient absorption. Consequently, this bacterium has evolved several mechanisms that allow it to adapt to pH variations. Another system different to urease is the periplasmic α-carbonic anhydrase, which contributes to buffering the pH of the cytoplasm and periplasm after the conversion of CO_2 from urea hydrolysis into HCO_3.

Furthermore, the ArsRS two-component signal transduction system is involved in pH sensing and in mediating changes in the expression of urease-encoding genes related to acid acclimation and pH homeostasis (15–17). A study published in 2018 by *Marcus et al*. found that at least 250 genes can be overexpressed or repressed in response to low pH (7,10).

In this study, we evaluated the effect of different pH values on the expression of *ureA* and *hopQ* genes in *H. pylori* strains with high virulence and low virulence profiles to understand their role in acid acclimation of strains with different virulence profiles.

METHODOLOGY

Bacterial strains and culture conditions

H. pylori type 1 [vacA (s1/m1)/cagA (+)] and type 2 virulence [vacA (s2/m2)/cagA (-)] isolates from the microorganism collection of the Bacteria & Cancer research group of the Faculty of Medicine of Universidad de Antioquia were previously identified by conventional PCR for the *ureA, cagA*, and *vacA* genes. They were cryopreserved in Brucella broth (BBL) ® supplemented with 20% glycerol and 10% fetal bovine serum. Bacteria were reactivated on plates containing Brucella agar (BBL) ® with 7% horse blood, Isovitalex ® (BBL) enrichment supplement, and they were incubated for a period of 10 days under microaerobic conditions (5% O_2 and 10% CO_2) at 37°C.

Exposure of *H. pylori* strains to acidic stress conditions

For exposure to acidic pH, subcultures of *H. pylori* strain ATCC 43504 with high virulence profile and isolate 439A with low virulence profile were used. Subcultures were collected from culture plates containing Brucella ® agar (BBL) and suspended in Brucella ® broth (BBL) supplemented with 10% fetal bovine serum and yeast extract (0.25%) to obtain a final suspension of approximately $6x10^8$ CFU/mL. The pH of the liquid culture was

adjusted by the addition of 1N HCl (EMSURE) to obtain pH values of 3, 4, and 5.0 considering the methodology described by Marcus *et al*. and Wen *et al*. The non-acidic pH used for all experiments was 7. Bacteria were suspended in 5 mL of Brucella ® broth (BBL) in filter culture flasks (SPL) ® and incubated under microaerophilic conditions at 120 rpm for one hour. This protocol was performed in triplicate.

RNA preparation

To isolate total RNA from bacteria exposed to low pH, *H. pylori* was harvested by centrifuging at 5000 x g for 5 min at 4ºC using the refrigerated centrifuge ROTANTA® MOD. 460. Total RNA was purified using the Rneasy® mini kit (Qiagen, Germany) following the manufacturer´s instructions and the RNA concentration was determined with the Nanodrop spectrophotometer (Thermo Scientific™) to be above 10 ng/µL.

cDNA preparation

A DNase treatment was previously performed by adding 2 µL of random hexamers to 10 µL of total RNA. RNA was converted to cDNA using the RevertAid™ H Minus cDNA kit (Thermo scientific ®); for which 4 µL of 5X reaction buffer, 1 µL of Ribolock RNase inhibitor, 2 µL of 10 mM dNTP mix, and 1 µL of RevertAid H Minus M-MuLV reverse transcriptase were added to a final volume of 20 µL. Finally, the mixture was incubated for 5 min at 25°C followed by 60 min at 42°C and the reaction was terminated by heating at 70°C for 5 min. The product of the reverse transcription reaction was stored at -80 °C.

Real-Time Polymerase Chain Reaction (qRT-PCR)

Semi-quantitative qRT-PCR was completed from cDNA using the Luna ® Universal Probe qPCR Master Mix and QuantiTect SYBR Green PCR Kit®, with a StepOnePlus™ Real-Time PCR System equipment (Applied Biosystems). All used genes were previously performed in the literature (Table 1).

Table 1. Primers for qPCR used in this study.

Primer	Sequence	Target gen	Reference
UreA-F	TGGATCATGCTTGCCACGCC	*UreA*	(18)
Urea-R	GCGGTAGCTTTGATTAGTGCCC		
HopQ-F	ATGGCACAAACTCAAAGACAAG	*HopQ*	(19)
HopQ-R	TAACACCGATCTCAACGCTAAA		
GyrA-F	TTTRGCTTATTCMATGAGCGT	*GyrA*	(20)
GyrA-R	CTCCATAAGAGCCAAAGCCC		

The preparation of the reaction was carried out with a final volume of 30 µL, adding 0.8 µL of each one of the primers, 10 µL of master mix, 10 µL of SYBR Green, 3.4 µL of water, and 5 µL of cDNA. The negative control contained the reaction mixture but no cDNA. qRT-PCR was completed using the standard cycling protocol, conditions were 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 60 seconds for 45 cycles.

Standard curve

To evaluate the efficiency and reproducibility of the amplification, a standard curve was constructed for all the genes from known concentrations of CFU/µL of *H. pylori*. For this purpose, isolates 576A and 512A (high virulence profile) as well as 545A and 583C (low virulence profile) were used for RNA extraction and cDNA synthesis. Subsequently, cDNAs from high and low virulence isolates were mixed to obtain a pool of isolate concentrations that were prepared with 1/10 serial dilutions. The standard curve included seven dilution points, each in duplicate.

Statistical analysis

The obtained results did not present a normal distribution due to the small number of samples used; therefore, non-parametric tests, such as the Mann-Whitney U-test, were

used to determine if there were significant differences between the pH analyzed and the *ureA* and *hopQ* genes.

RESULTS

Real-time PCR amplification efficiencies and linearity

Real-time PCR efficiencies were calculated from the equation E = 10[-1/slope]. The points on the standard curve were fitted to a straight line by linear regression. From the slope of the standard curve, the efficiency of the amplification reaction was determined, and linearity was calculated from the correlation coefficient R^2 obtained in linear regression. All transcripts showed high efficiency rates, for *hopQ* (115%), *gyrA* (112%) and *ureA* (100%) with high linearity (Pearson's correlation coefficient r > 0.95) (Table 2).

Table 2. Efficiency and linearity of genes

Gen	Efficiency (%)	R^2
hopQ	115	1
ureA	100	0,99
gyrA	112	0,99

H. pylori gene expression exposed to different values of pH.

To identify important changes in the expression of *ureA* and *hopQ* genes in response to low pH, it was necessary to ensure that *H. pylori* isolates remained viable throughout the experiment. To determine their viability, three experiments were performed on separate days using Brucella agar at pH 7.0 as control. To demonstrate the ability to grow in different acidic pH values, Brucella broths containing the isolates were incubate for 1 hour, then 10 µL of Brucella broth were resuspended in the solid medium. The expression patterns for both genes are shown in Figures 2 and 3 after calculating the RQ from the delta CT obtained for each pH and the efficiency obtained for the genes of interest.

For the *ureA* gene, an increase in expression was observed for the high virulence isolate compared to pH 7, being higher at pH 4 and 3, while in the low virulence isolate, although an increase in *ureA* gene expression was observed as pH decreased, it did not exceed the expression shown at pH 7 (Figure 2).

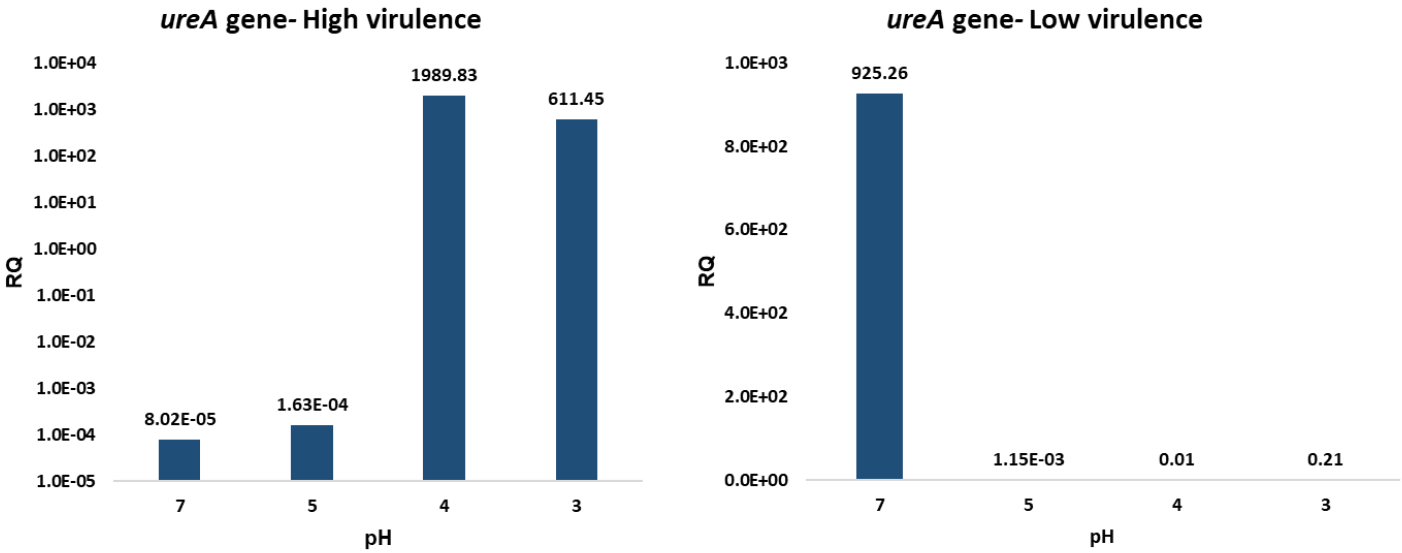

Figure 2. Comparison of susceptibility of high and low virulence strains of *H. pylori* after exposure to pH 7, 5, 4 and 3 for one hour. RQ indicates the difference in *ureA* gene expression levels after exposure to acidic pH.

On the other hand, for the *hopQ* gene, a decrease in expression was observed at pH 5 and 4 in the low virulence isolate, while gene expression at pH 3 had a similar behavior at pH 7. Although for the high virulence isolate, an increase in gene expression was observed at the different acidic pH with respect to pH 7. The gene expression was not significant since the RQ was not greater than 1 (Figure 3).

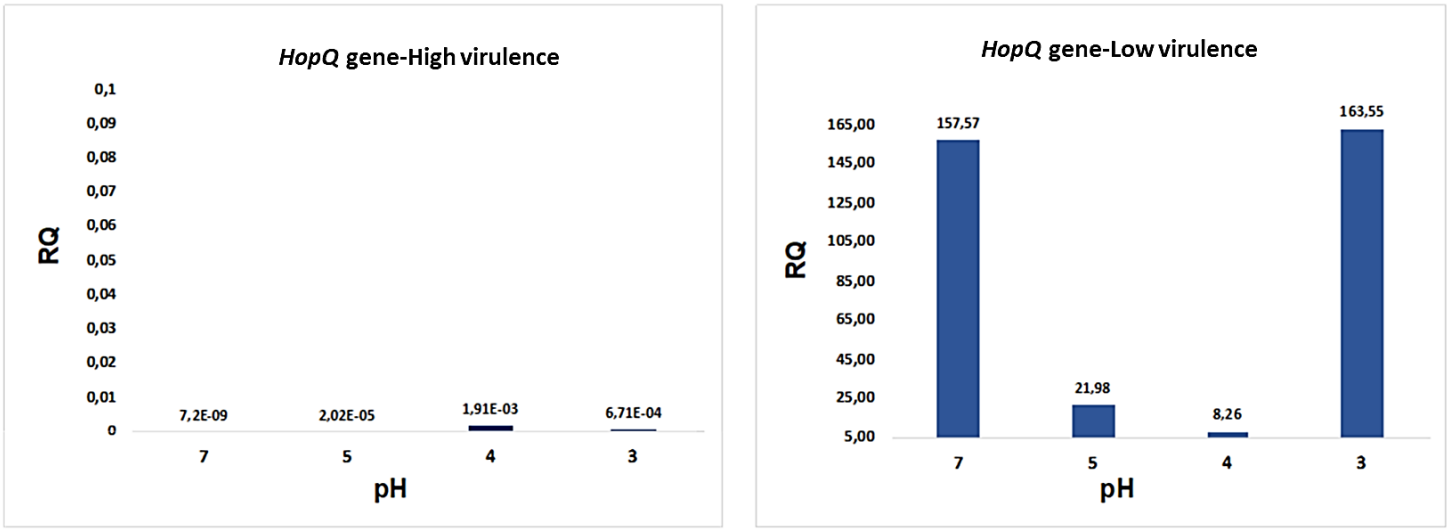

Figure 3. Comparison of susceptibility of high and low virulence strains of *H. pylori* after exposure to pH 7, 5, 4, and 3 for one hour. RQ indicates the difference in *hopQ* gene expression levels after exposure to acidic pH.

In addition, significant differences were observed between isolates with respect to *hopQ* gene expression, which was higher in the low virulence isolate with a value of $P < 0.05$. The *ureA* gene did not show significant differences, which may be due to the small number of samples used or due to the role of this gene in maintaining the physiology of the bacterium, which presents less genetic variability and therefore was more conserved. Unlike the OMP that are directly exposed to the environment and produced greater genetic variability. However, it is observed that gene expression tends to be higher in the isolate of high virulence when subjected to acid stress.

DISCUSSION

Colonization of the stomach represents a challenge for *H. pylori* since it must adapt to the fluctuations of the gastric environment and not only survive, but also grow and replicate at very acidic pH levels where other types of microorganisms could not easily adapt. This ability of *H. pylori* to adapt to the variation of stomach pH is due to the

regulation in the expression of some genes. These genes are directly affected by pH and their transcription levels are higher when *H. pylori* is subjected to acid stress. Previous studies have shown significant differences in the expression patterns of *H. pylori* genes, including the genes selected for this study. *Wen Y et al., Sachs G et al.,* and *Merrell DS et al*. have concurred that the *ureA* and *OMP* genes increase and decrease their expression, respectively, in the presence of acid stress (10,11,21). Similarly, *H. pylori* is constantly exposed to pH fluctuation during its colonization of the gastric epithelium.

Among the genes reported to positively regulate their expression in the presence of acid pH are those belonging to the enzyme urease, being ureA/B structural components of the enzyme. *Bijlsma et al*. have shown that this enzyme is crucial for the survival of *H. pylori* under acidic conditions since at pH below 7 there is an induction of its expression. Furthermore*, Marcus E et al.* found that the *ureA* gene is significantly increased in expression at pH 6 compared to pH 7.4; even for the *ureB* gene, increased expression was observed at pH 4 compared to pH 3 and 6 (7, 22).

These findings agree with the results obtained for strain ATCC 43504, which present a high virulence profile where a gradual increase in the expression of the *ureA* gene was observed with respect to pH 7, while pH 4 was the factor that produced the greatest induction of the gene. However, an intriguing pattern is observed for isolate 439A with a low virulence profile where the expression levels for the *ureA* gene are lower than 1 RQ at acidic pH compared to pH 7, which presents an expression of approximately 925 RQ. This result suggests that the *ureA* gene is not a key factor in survival under acidic conditions for *H. pylori* strains with low virulence profile, these strains may use other adaptive mechanisms such as periplasmic α-type carbonic anhydrase (α-CA) or the ArsRS system, which responds to acid stress conditions when the urease system is not sufficient for colonization of the stomach (15,17).

On the other hand, among the OMPs, there is hopQ, which is believed to be fundamental in the adhesion of *H. pylori* to gastric epithelial cells and whose expression decreases in the presence of acidic pH. This is an environmental signal that causes changes in the adhesion properties of the cell (9,11). Similarly, to what occurs with the *ureA* gene, differences were found in the expression patterns of *hopQ*. In this case, the isolate of high virulence presents an expression level for the *hopQ* gene lower than 1 RQ. However, as pH decreases the expression of the gene tends to increase, although not significantly. In turn, the low virulence isolate expresses much higher levels for this gene that decreases as pH decreases. Nevertheless, at pH 3 an induction of its expression is observed reaching levels similar to those expressed at pH 7. This result agrees with that published in 2018 by *Marcus EA et al*, where they reported an induction of expression for this gene >1.5-fold, where it is stated that the differences in the results with other studies may be due to differences in experimental conditions such as the strains used the method of analysis and the time of exposure to acidic conditions (7).

The obtained results demonstrate the capacity of *H. pylori* to colonize the gastric epithelium through mechanisms of adaptation to acid pH that favor not only transcription of genes involved with acid acclimation such as *ureA* but also genes that participate in the colonization process. However, it is likely that the way in which *H. pylori* adapts to these conditions differs depending on the virulence of the strain. This indicates that not only there are significant differences in the expression of virulence genes, but also a possible correlation between virulence and the mechanisms that *H. pylori* uses to survive acid stress conditions.

Although the urease enzyme and associated genes are one of the main mechanisms reported for the adaptation of *H. pylori* to acidification conditions, it was evidenced that this mechanism acquires greater relevance for strains with type 1 virulence. Consequently, it can be inferred that the association between the development of gastroduodenal diseases, gastric cancer, and the expression of the *cagA*+ and *vacA*

s1m1 genotypes are due to a greater predilection to remain in the gastric mucosa in contact with epithelial cells where cell-mediated mechanisms exist to maintain a more basic pH compared to other regions such as the gastric lumen. Thus, exposure to more acidic environments suggests being a factor that triggers a higher transcriptional response for strains of high virulence compared to strains expressing a different virulence, which may be more adapted to stay in more acidic environments or use different mechanisms to survive this stress condition (24,25).

Finally, although qPCR is not a routine diagnostic method, it is a useful test to detect not only the presence of *H. pylori* but also its viability, being a promising method, especially in patients where other diagnostic methods such as culture present lower sensitivity rates due to the use of antibiotics or Protons Pump Inhibitor that can inhibit the isolation of the microorganism.

CONCLUSIONS

The differences found in the expression of the genes evaluated between high and low virulence isolates indicate the importance of carrying out a more detailed study involving the analysis of other *H. pylori* genes, which could contribute to the understanding of the pathophysiology of the disease caused by this microorganism. Furthermore, the results showed that in these two isolates with different virulence profiles there is an important effect of pH on the regulation of the expression of the *ureA* and *hopQ* genes. This possibly indicates that the change in pH will condition the adaptation mechanisms of each strain or isolate to the conditions of the gastric environment.

ABBREVIATIONS

H. pylori	*Helicobacter pylori*
MALT	Mucosa Associated-Lymphoid Tissue lymphoma
cagA	Cytotoxin associated gene A
vacA	Vacuolating cytotoxin A
OMP	Outer Membrane Proteins

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declared no potential conficts of interest.

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Availability of data and materials

All data generated or analyzed during this study are included in this manuscript.

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