The Absence of Urease Enzymatic Activity of *Helicobacter pylori* Coccoid Form

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ABSTRACT

Background: *Helicobacter pylori (H. pylori) is a gram negative and pleomorphic bacteria that able to change its morphology according to the environment. The objective of the study was to determine the biochemical and some genetic characteristic of coccoid form of H. pylori induced by starvation, aerobiosis and antibiotic.*

Method: *The material of the study is an isolate of spiral form of CagA positive H. pylori grown from gastric biopsy specimen of a patient with chronic gastritis. The CagA positive isolate was subcultured in liquid media containing the sheep sera. The sample was divided into three groups each group consist of 27 tube. Each tube contained 10^9 CFU of H. pylori bacteria/ml in 4 ml liquid media. So the experiment was performed in 3 replicates. In the first group of sample, coccoid form was induced by a prolonged culture under microaerophilic condition without the addition of fresh media, in the second group by aerobiosis, while in the third group by addition of 0.1 ì g amoxycillin/ml cultured in microaerophilic condition. Periodic sampling was done every day to calculate the percentage of coccoid form, to observe the possibility to regrow the spiral form and for serial electron microscopic observation. One tube is picked up in every periodic sampling. In tubes containing antibiotic the periodic sampling was done one hourly. Detection of cagA and ureA gene was done by Polymerase Chain Reaction (PCR) with appropriate primers.*

Results: *The time needed for the development of coccoid form: Length of time from the start of the experiment needed to reach 100% coccoid form was: 49 days in microaerophilic with starvation, 28 days in aerobiosis with starvation, and 13.5 days in antibiotic. Result of biochemical test: Urease enzymatic activity was only positive in spiral form. All samples of coccoid form due to all the 3 stressors did not show any urease enzymatic the activity. PCR of ureA gene: All samples of spiral and coccoid form showed positive band of ureA gene and cagA gene. Western blot of protein CagA, urease A and urease B: Western blot analysis showed that in spiral form and all coccoid form band of urease A and urease B is clearly seen,while cagA in Western blot only clearly seen in spiral form but it is absent in cocoid form.*

Conclusion: *Troughout the cycle of coccoid form the urease gene responsible for the production of urease and cagA gene responsible for virulence was in intact condition. However, despite the presence of urease protein in coccoid form the urease enzymatic activity was absent. This fact has several diagnostic and clinical implications.*

Keywords: *urease enzymatic activity, coccoid form, Helicobacter pylori*

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INTRODUCTION

Helicobacter pylori (H. pylori) is a gram negative and pleomorphic bacteria that able to change its morphology according to the environment. In a good environmental condition the bacteria is in spiral or curved form but in a bad condition the spiral form change in to coccoid form. The habitat of *H. pylori* is

in the antrum of the stomach and caused chronic gastritis, peptic ulcer and even gastric cancer.[1,2] The change form spiral to coccoid form is stimulated by environmental stresses including lack of nutrition,[3] antibiotics,[4] prolonged incubation,[5] extreme pH and temperature.[6]

Epidemiologic studies showed *H. pylori* infection can be transmitted by fecal-oral route especially in the developing countries, associated with bad hygiene and sanitation.[7,8] Theoretically most of *H. pylori* bacteria in the environment is in coccoid form, so the most likely coccoid form is responsible for the transmission of *H. pylori* through environment. In the other hand it was known that it is very difficult to grow coccoid in vitro in the laboratory. But experimental study showed that coccoid form which is unculturable in the laboratory was culturable in the stomach of mice. It's mean that coccoid form can be infectious.[5] Studies showed that coccoid form is more resistant to antibiotic compared to spiral form.[9]

Despite the importance of coccoid form in transmission of *H. pylori* from environment, the biology of *H. pylori* is still obscure. To study the coccoid form in natural condition is very difficult. Therefore the experimental or artificial development of coccoid form by various known stressor is a very important method to study the biology of coccoid form. The knowledge of the biology of *H. pylori* coccoid form is very important because the possible diagnostic and clinical implication.

OBJECTIVE

The objective of the study was to determine the biochemical and some genetic characteristic of coccoid form of *H. pylori* induced by starvation, aerobiosis and antibiotic. This study for is a part of a bigger study to search the several characteristic of coccoid form induced by starvation, aerobiosis and antibiotic including ultramicroscopic features.[10]

METHOD

The material of the study is an isolate of spiral form of cytotoxin CagA positive *H. pylori* grown from gastric biopsy specimen of a patient with chronic gastritis. The biopsy specimen was cultured in BAP (Blood Agar Plate) added with Dent's Supplement and Vitox. The bacteria from grown colony were identified microscopically and biochemically. After identification the *H. pylori* isolate was examined for cagA using Polimerase Chain Reaction (PCR). The CagA positive isolate was subcultured in liquid media containing the sheep sera. The sample was divided into three groups each group consist of 27 tubes. Each tube contained 10^9 colony forming units (CFU) of *H. pylori* bacteria/ml in 4 ml liquid media. So the experiment was performed in 3 replicates. In the first group of sample, coccoid form was induced by a prolonged culture under micro-aerophilic condition without the addition of fresh media, in the second group by aerobiosis, while in the third group by addition of antibiotic and cultured in micro-aerophilic condition. Microaerophilic condition was acquired using CO_2 incubation in 10% CO_2 concentration. Aerobiosis was a culture using incubator with room air oxygen condition. Antibiotic condition was microaerophilic culture with the addition of 0.1 microgram amoxycillin/ml. Periodic sampling was done every day to calculate the percentage of coccoid form, to observe the possibility to regrow the spiral form and for serial electron microscopic observation. One tube is picked up in every periodic sampling. Instead of every day in starvation group and aerobiosis group for tubes containing antibiotic the periodic sampling was done every hour. The serial transmission electron microscopic study was done with JEM.10-JEOL using Sabatini method in the Eijkman Institute of Mollecular Biology, Jakarta.[11]

Stages of Coccoid Form

Spiral stage (SP) was the stage beginning with the start of the experiment until 100% of bacteria was in coccoid form. Viable and culturable (VC) stage was the stage starting with 100% coccoid until the bacteria can not be cultured further. Viable non-culturable (VNC) stage was the stage between the start of unculturability until the appearance of signs of coccoid death as observed microscopically and ultra-microscopically. The stage after that was called non viable stage (NV). With the periodic sampling the time needed for the formation of 100% coccoid was known.

Biochemical Characteristics

From each stage of *H. pylori* (SP, VC, VNC, and NV) 9 tubes were picked up for biochemical test consisted of urease, catalase, oxidase, glucose, lactose, sucrose, maltose, and manitol. All samples was also examined for motility, Simmon citrate test, and Triple Sugar Iron (TSI) test. Urease activity was tested according to method published in the literature.[12]

Detection of cagA and urease A gene

Detection of cagA and ureA gene was done by PCR technique from Narikawa et al,[13] with slight modification. At first 1 ml of liquid culture of *H. pylori* was put into a centrifuge tube. The bacteria was washed and sediment using phosphate-buffered saline (PBS) pH 7.2 in 11,000 g. Deoxyribonucleic acid (DNA) was extracted using DNA Zol (Invitrogen) genomic DNA collected from the extraction was resuspended in TE buffer (10 mM Tris hydrochloride and 1 mM EDTA (pH 8.0). The total quantity of the extracted DNA was measured with DyNA Quant

(Hreffer Pharmacia Biotech, USA). The sequence of primer for the detection of cagA was 5'ATAATGCTAAATTAGACAACTTGAGCGA and 5'TTAGAATAATCAACAAACATCACGCCAT. The sequence of primer in the detection of ureA was 5'GCCAATGGTAAATTAGTT and 5'CTCCTTAATTGTTTTTAC.[14] Amplification reaction was performed in 50 uL using PCR Core System (Promega, USA) in MyCycler machine (Biorad, USA) with the condition as following. Denaturation 94°C for 1 minute, annealing 72°C for 1 minute, extension 72°C for 1 minute each for 35 cycle. The detection of amplification product was performed in 2% agarose gel with ethidium bromide staining and UV light. cagA band was 298 bps and ureA band was 411 bps. From every stage of *H. pylori* beginning from spiral form until non viable coccoid, a sample was picked for the PCR. For comparison the thickness of bands of DNA observed in the DNA electrophoresis was divided into three categories: +3 the thickest band, +1 the thinnest band seen and +2 if the DNA band has the thickness between category 1 and category 3.

Protein Composition Analysis

Protein composition of spiral form and each stage of coccoid form were done with SDS PAGE 10% with Coomasie Briliant Blue staining using standard of molecular weight high range protein (Invitrogen).

Western Blotting

The electrophoresis protein in the polyacrilamide gel was transferred to polyvinylidene fluoride (PVDF) membrane using transfer buffer. The PVDF was blocked with TSBT and skim milk. The transfer was done using minitransblot (Biorad) using 100 V power in 60 minutes later the PVDF membrane was blocked with antibody 1 (mouse monoclonal antibody to UreA, UreC and cagA) and washed 3 times using PVDF. Later the membrane was blocked with antibody 2 (secondary anti mouse antibody) for 2 hours in room temperature. The PVDF membrane was soaked in 4 CN substrate solutions for 10-15 minutes until the bands was developed. The reaction was stopped using aquadest.

RESULTS

The Time Needed for The Development of Coccoid Form

Length of time from the start of the experiment needed to reach 100% coccoid form was: (1) Microaerophilic 49 days, (2) Aerobiosis 28 days, (3) Antibiotic 13.5 days. The information in detail is shown in the table 1.

Different length of time was required for transformation from spiral to reach 100% of coccoid form. The viability of the coccoid form was the longest in microaerophilic with starvation condition and it was the shortest in antibiotic condition.

Here we can see that in the group with starvation the stage of viable and culturable was only 9 days and after that the coccoid become unculturable the although still living. Forty nine days later the coccoid become nonviable or died. In contrast, in antibiotic group the culturability was only 3.5 days and after that the coccoid is no longer culturable although it is still viable, and 13.5 days latter the coccoid ied.

Table 1. Formation of coccoid form of *H. pylori* in variable stress condition

Code	Length of Time for the development of coccoid form from spiral form								
	Microaerophilic + Starvation			Aerobiosis			Antibiotic		
	Length of Time	% Coccoid	Culturability	Length of Time	% Coccoid	Culturability	Length of Time	% Coccoid	Culturability
SP	2 days	0	+	2 days	0	+	2 days	0	+
VC	9 days	98.13	+	8 days	99.99	+	84 hours (3,5 days)	99.93	+
VNC	49 days	100	-	28 days	100	-	324 hours (13,5 days)	100	-
NV	80 days	100	-	48 days	100	-	670 hours (± 28 days)	100	-

SP: Spiral; VC: Viable Culturable; VNC: Viable Non Culturable; NV: Non Viable

Table 2. The results of biochemical test on spiral and coccoid form of *H. pylori*

Stressor	Code	N	Ure	Cat	Ox	Glu	Lac	Mal	Man	Mot	S.cit	TSI
	SP	9	+	+	+	-	-	-	-	+	-	b/b -/-
Microaerophilic with	VC	9	-	+	+	-	-	-	-	-	-	b/b -/-
starvation	VNC	9	-	+	-	-	-	-	-	-	-	b/b -/-
	NV	9	-	+	-	-	-	-	-	-	-	b/b -/-
	VC	9	-	+	+	-	-	-	-	-	-	b/b -/-
Aerobiosis	VNC	9	-	+	+	-	-	-	-	-	-	b/b -/-
	NV	9	-	+	-	-	-	-	-	-	-	b/b -/-
	VC	9	-	+	+W	-	-	-	-	-	-	b/b -/-
Antibiotic	VNC	9	-	+	-	-	-	-	-	-	-	b/b -/-
	NV	9	-	+	-	-	-	-	-	-	-	b/b -/-

W: Weak

Result of Biochemical Test

From the table 2 it can be seen that the urease enzymatic activity was only positive in spiral form. All samples of coccoid form due to all the 3 stressors did not show any urease enzymatic the activity. In the other hand the result of the catalase test was positive in all stages of coccoid form.

PCR of gene ureA

Following is the table 3 showed this descriptive data of ureA gene band according to the stressors.

Table 3 showed that all samples of spiral and coccoid form showed positive band of ureA gene. It has the meaning that the ureA gene is still intact during the development of coccoid form. The thickest band was found in VC coccoid followed by viable was culturable (VNC) and nonviable (NV) coccoid.

PCR of gene cagA

Table 4 shows that in all isolate of coccoid form showed band of cagA gene, meaning that throughout the development of coccoid form the cagA gene was intact. From table 4 we can so that the band thickness of cagA gene was the thickest in VC coccoid followed by VNC and NV coccoid.

Table 3. The descriptive data of gene ureA band in coccoid form of *H. pylori* formed after various stressors

Depend. Variable	N	Stressor	Band Thickness			
			Mean	Standard Deviation	Minimum	Maximum
Gene UreA Coccoid VC	9	Microaerophilic + starvation	2.78	0.44	2.00	3.00
	9	Aerobiosis	2.67	0.5	2.00	3.00
	9	Antibiotic	2.33	0.5	2.00	3.00
Gene UreA Coccoid VNC	9	Microaerophilic + starvation	2.33	0.71	1.00	3.00
	9	Aerobiosis	2.33	0.71	1.00	3.00
	9	Antibiotic	1.89	0.93	1.00	3.00
Gene UreA Coccoid NV	9	Microaerophilic + starvation	2.22	0.44	2.00	3.00
	9	Aerobiosis	2.22	0.44	2.00	3.00
	9	Antibiotic	1.56	0.73	1.00	3.00

Table 4. The descriptive data of gene cagA band in coccoid form of *H. pylori* formed after various stressors

Depend. Variable	N	Stressor	Band thickness			
			Mean	Std. Deviation	Minimum	Maximum
Gene cagA Coccoid VC	9	Microaerophilic + starvation	3.00	0	3.00	3.00
	9	Aerobiosis	2.67	0.5	2.00	3.00
	9	Antibiotic	1.67	0.71	1.00	3.00
Gene cagA Coccoid VNC	9	Microaerophilic + starvation	1.00	0	1.00	3.00
	9	Aerobiosis	2.67	0.5	2.00	3.00
	9	Antibiotic	1.56	0.88	1.00	3.00
Gene cagA Coccoid NV	9	Microaerophilic + starvation	-	-	-	-
	9	Aerobiosis	2.22	0.44	2.00	3.00
	9	Antibiotic	1.33	0.5	1.00	2.00

Protein analysis of *H. pylori* spiral and coccoid form

Following (figure 1) is the result of SDS PAGE of protein in *H. pylori* from spiral form and coccoid form induced by prolonged starvation. In the picture we can see that in electrophoresis taken from coccoid form we can identify protein bands of *H. pylori*, such as 120 kDa, 86 kDa band, 67 kDa, 29 kDa, 26 kDa, and 18 kDa. Band of 120 kDa which is very clear in the spiral form is very thin in coccoid form. Band of 26 kDa dan 18 kDa that looks very clear in the coccoid form is very thin in spiral form. Protein urease A was seen as 29 kDa band while protein urease B was seen as 67 kDa band, and both bands can be identified clearly meaning that urease proteins were present. In figure 2 the SDS PAGE of *H. pylori* spiral and coccoid form induced by aerobiosis was sho wn. Here too we can identify clearly the 26 kDa band of urease *H. pylori* 67 kDa of urease B protein.

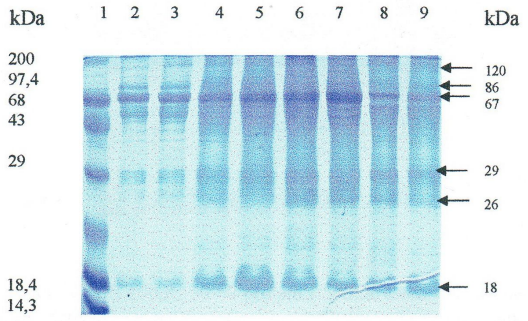

Figure 1. Analysis of SDS PAGE 10% of *H. pylori* with starvation as stressor. Lane 1: standard, Lane 2 and 3: spiral form, Lane 4 and 5: VC coccoid, Lane 6 and 7: VNC coccoid, Lane 8 and 9: NV Coccoid

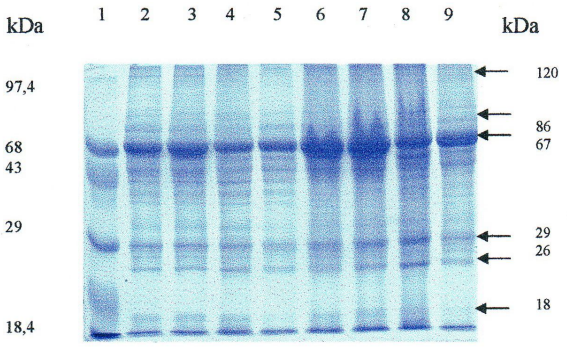

Figure 2 show the SDS PAGE of spiral and coccoid form induced by antibiotic. Here we can see the similar pattern with figure 1 and figure 2. Note the presence of 67 kDa and 29 kDa band of urease band urease A.

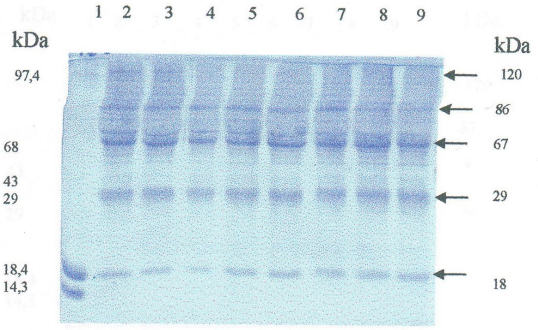

Figure 3. Analysis of SDS PAGE 10% of *H. pylori* with antibiotic stressor. Lane 1: standard, Lane 2 and 3: Spiral form, Lane 4 and 5: VC coccoid, Lane 6 and 7: VNC coccoid, Lane 8 and 9: NV coccoid

Western Blot of protein CagA, urease A and urease B

Western Blot analysis (see figure 4) using primary mouse antibody and secondary anti mouse antibody (anti-cagA, anti-urease A and anti-urease B). It showed that in spiral form and all coccoid form band of urease A and urease B is clearly seen, while cagA in Western blot only clearly seen in sp iral form but it is absent in cocoid form.

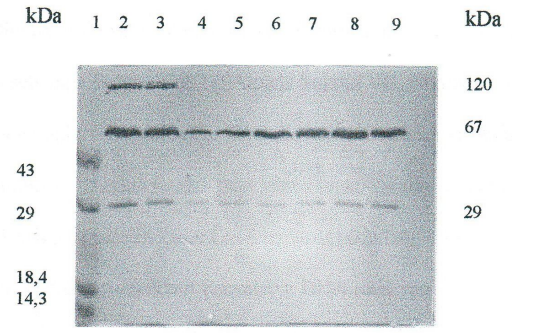

Figure 4. Analysis of Western blotting of CagA protein, urease A and urease B. Lane 1: standard, Lane 2 and 3: spiral form, Lane 4: VC coccoid with aerobiosis, Lane 5 : VC coccoid with antibiotic stressor, Lane 6: VNC coccoid with aerobiosis, Lane 7: VNC coccoid with antibiotic, Lane 8: NV coccoid with aerobiosis, Lane 9: NV coccoid with antibiotic

DISCUSSION

This study is a prospective experimental study on coccoid form of *H. pylori* using three different stresses, starvation, aerobiosis, and antibiotics. We can see that in this experiment in the beginning a coccoid was still culturable in vitro. So in the real situation if due to a stress, for example Proton Pump Inhibitor (PPI) or antibiotic the spiral form can change partially or totally into spiral form. But they can revert into spiral form again if the stress is terminated for example by stopping the drug as long as the coccoid is still in stage of VC. From this study it is clear that in the development of coccoid form due to all 3 stressors, despite the intact urease A gene and the presence of

urease protein in the coccoid form, there is no enzymatic activity of the urease. The cause of this phenomenon is not known. This is the first report of the absence of urease enzymatic activity in the coccoid form induced by starvation, aerobiosis and antibiotic. The other report of She FF et al, mentioning that in the coccoid form induced by metronidazole the urease activity drastically reduced, beside the reduced concentration of urease protein.[15] In the other report she also showed that coccoid form induced by tape water also showed reduced urease activity compare with spiral.[16]

This fact has several diagnostic and clinical implications. It was known since long time that if we will use Campylobacter Like organism (CLO) after endoscopic biopsy it is better if the patient was told to avoid antibiotic or PPI several days before endoscopy. The same thing also happens to patients in the preparation for a Urea Breath Test (UBT). If the preparation is not done properly there is a possibility of false negative CLO or UBT. In the report of Manes et al the administration of 20 mg omeprazole daily for 1 week will result in 20% false negative UBT while the same dose given for 2 weeks resulted in 24% false positive UBT after the drug is stopped for 2 week the UBT become 100% positive again.[17,18] Also, if we want to evaluate the result of *H. pylori* eradication regiment, we must wait for one month after the last dose of antibiotic. The evaluation done before one month after eradication can result in the negative of UBT or CLO. Why one month? The reason of this phenomena is not fully understood, but it is very likely that after antibiotic or PPI if the bacteria is not killed and some can escapes the eradication and change into the coccoid form that does not show enzymatic activity of urease. One month is may be the time needed for the coccoid form to reverse to spiral form again, and show enzymatic activity again.

CONCLUSION

With the artificial induction of *H. pylori* coccoid form followed prospectively we can study the coccoid form of *H. pylori* in all three stages starting with viable culturable, viable nonculturable, and nonviable. Troughout the cycle of coccoid form the urease gene responsible for the production of urease and cagA gene responsible for virulence was in intact condition. However, despite the presence of urease protein in coccoid form the urease enzymatic activity was absent. This fact has several diagnostic and clinical implications. In all situation in which there is a possibility of the conversion of *H. pylori* spiral form to coccoid form such as the administration of antibiotic or may be PPI, the diagnostic based on the urease enzymatic activity should not be used because a possibility of false negative result of the test. This study showed that all form of coccoid bacteria of *H. pylori* does not show urease enzymatic activity. The use of UBT and CLO should be avoided before stopping of antibiotic or PPI at least for two weeks. The interval free of antibiotic or PPI maybe needed for the coccoid form to reverse into the spiral form again.

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