대한임상미생물학회지 : 제4권 제2호 2001
Korean J Clin Microbiol 2001 ; 4 : 102-107

타액에서 Nested Polymerase Chain Reaction을 이용한 *Helicobacter pylori* 검출

한태희, 백인기

인제대학교 상계백병원 임상병리과

Nested Polymerase Chain Reaction Assay of *Helicobacter pylori* in Saliva

Tae Hee Han, M.D., and In Ki Paik, M.D.

Department of Clinical Pathology, Inje University Sanggye Paik Hospital, Seoul, Korea

Background : The aim of this study was to investigate the prevalence of *Helicobacter pylori* in the saliva of infected patients and the relation between *H. pylori* in the saliva and the severity of gastric infection.

Methods : Active gastric infection was determined by the ^{13}C-urea breath test. Bacteria in saliva were detected by the nested polymerase chain reaction, using primer sets EHC-U/EHC-L and ET-5U/ET-5L.

Results : The PCR assay was able to detect as few as 5 *H. pylori* /mL. A total of 82 (71.9%) out of 114 patients with gastroduodenal diseases were positive by ^{13}C-urea breath tests. Among these 82 patients, 21 (25.6%) were PCR positive in their saliva. *H. pylori* was present in the saliva of patients with highly active gastric infections (>50 ‰). No *H. pylori* was detected in saliva of patients with no active gastric infections.

Conclusions : In patients with highly active gastric infections, *H. pylori* may be transmitted via saliva. The PCR assay of *H. pylori* in saliva is not useful for detecting gastric infection but may be a useful tool for the screening of highly infectious patients.

(Korean J Clin Microbiol 2001;4(2):102-107)

Key words : *Helicobacter pylori*, Polymerase chain reaction, Saliva

INTRODUCTION

Helicobacter pylori is recognized as an important cause of active chronic gastritis in humans and it plays a significant pathogenic role in the development and recurrence of gastric and duodenal ulcer, and gastric cancer [1-4].

The human stomach is considered as the primary reservoir of this microorganism. Because *H. pylori* has been recovered from gastric juice, regurgitation and vomiting could provide obvious sources of transmission[5]. However, there is considerable debate about the transmission and the source of this infection[5]. Ever since *H. pylori* was successfully isolated by culture from the oral cavity of a patient in 1989[6], the oral cavity has received attention as a possible reservoir of *H. pylori* and an oral to oral mode of transmission has been postulated[7,8]. In addition continued presence of *H. pylori* in the oral cavity may be an important source of gastric recurrence after eradication attempts[7,9].

Because *H. pylori* may be transmitted by saliva to other individuals in an oral to oral transmission mode, the detection of this bacterium in saliva has been an important concern[6-18]. However, the isolation of *H. pylori* in saliva by culture is complicated and time-consuming[6-11]. Recently polymerase chain reaction assays have proved to be

접수번호 : CM 4-02-03
교 신 저 자 : 백인기
(139-707) 서울 노원구 상계7동 761-1
인제대학교 상계백병원 임상병리과
Tel : 02)950-1227 Fax : 02)950-1244
E-mail:ikpaik@sanggyepaik.or.kr

highly sensitive and specific tools and are regarded as the method of choice for detecting *H. pylori* in saliva[12-18].

The aim of this study was to investigate, using the sensitive and specific PCR assay, the prevalence of *H. pylori* in the saliva of infected patients and the relation between *H. pylori* in the saliva and the severity of gastric infection.

MATERIALS AND METHODS

Subjects and samples

From March 1999 to June 2000, 114 patients, who underwent upper gastrointestinal endoscopy due to dyspeptic complaints and who were subsequently diagnosed with gastroduodenal diseases, were enrolled in this study. The patients who had no history of antibiotic treatment for at least one month prior to the enrollment, were selected. Gastric infection with *H. pylori* was determined by the ^{13}C-urea breath test. Before the ^{13}C-urea breath test, patients were asked to expectorate saliva directly into new sterile containers. The saliva was stored at -70℃ until DNA extraction.

Table 1. Summary of the results for the ^{13}C-urea breath test and *H. pylori* PCR assay

UBT (‰)	PCR		
	Negative	Positive	Total(%)
Neagative (< 4)	35	0	35(30.7%)
Positive (≥4)	58	21	79(69.3%)
Total (%)	93(81.6%)	21(18.4%)	114(100%)

UBT, ^{13}C-Urea breath test.

^{13}C-urea breath test

The initial breath sample was collected in a container (Helikit, Isodiagnostika, Alberta, Canada). Then patient drank 75 mL of water containing 75 mg ^{13}C-urea. The second breath sample was collected 30 minutes thereafter. The breath samples were analysed with HeliView (Medichems, Seoul, Korea). If the ratio of $^{13}CO_2$ to $^{12}CO_2$ between the baseline and the second samples increased by greater than 4 deltamillipercent (‰), ^{13}C-urea breath test was positive and the presence of *H. pylori* in stomach was defined[19].

Preparation of Genomic DNA for PCR assay

In order to remove cell debris, 2 mL of saliva or cultivated *H. pylori* (clinical isolates) suspension was passed through 3.0 μm filter (Milipore, Bedford, MA, USA). The filtrate was centrifuged in a table-top centrifuge for 20 minutes at 10,000 g. The supernatant was removed and 1 mL 5% Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA, USA) was added. The tube was incubated in a thermostat (SeouLin Bioscience, Seoul, Korea) for 20 min at 99℃. After cooling, tubes were vortexed briefly and centrifuged. The supernatant was used as a template DNA in the subsequent PCR reaction.

PCR primers

The primer set of EHC-U (5' -CCC TCA CGC CAT CAG TCC CAA AAA-3') and EHC-L (5' -AAG AAG TCA CGC CAT CAG TCC CAA AAA-3') produces 417-bp PCR product. The region targeted by the primers EHC-U/EHC-L is located in 80,076-80,492 bp of the genome of H. pylori. Additional primer set of ET-5U (5' -GGC AAA TCA TAA GTC CGC AGA A-3') and ET-5L (5' -TGA GAC TTT CCT AGA AGC GGT GTT-3'), internal to the fragment amplified by EHC-U/EHC-L, produce 230-bp PCR product.

Table 2. Summary of test results of for the PCR of saliva and ^{13}C-urea breath test according to diagnosis

Diagnosis	PCR of saliva		UBT	
	Negative	Positive	Negative	Positive
Duodenal ulcer	27	5	9	23
Gastric ulcer	15	6	5	16
Gastritis	19	4	5	18
Gastritis & duodenal ulcer	32	6	16	22
Total (n=114)	93(81.6%)	21(18.4%)	35(30.7%)	82(69.3%)

UBT, ^{13}C-urea breath test; UBT Negative,〈 4 ‰; UBT Positive, ≥4 ‰.
Neither PCR nor UBT was significantly correlated with diagnosis (P=0.572, P=0.377)

The region targeted by the primers ET-5U/ET-5L is located in 80,198-80,427 bp of the genome of *H. pylori*[12].

PCR amplification

Amplification was performed in 20 μl reaction mixture containing the following: 0.2 μM of each primer (EHC-U/EHC-L); 200 μM (each) dATP, dCTP, dTTP, and dGTP; 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 40 mM KCl; 1 unit of Taq polymerase (Perkin-Elmer, Cetus, CT, USA); and 1 μl of DNA sample. Forty cycles of amplification were preformed in a DNA thermal cycler (GeneAmp PCR 9600 system, Perkin-Elmer, Cetus, Connecticut, USA). Each cycle consisted of a 45 seconds denaturation step at 95℃, a 45 seconds annealing step at 59℃, and a 30 seconds extension step at 72℃. The final cycle included an extension step (10 minutes at 72℃) to ensure full extension of the product. In nested PCR with the other primer set (ET-5U/ET-5L), the profile was similar to the first PCR, with the exception that 0.2 μl of the first PCR product served as the template for the second PCR. In addition, only 25 amplification cycles were used. DNA extracted from *H. pylori* (clinical isolate) served as a positive control. For the negative control, normal saline was used. Positive and negative control reactions were included in each batch of amplifications.

Analysis of PCR amplified products

The products of PCR were analysed by electrophoresis on 1.5% agarose gel and ethidium bromide staining. A band at 230bp was considered positive PCR result.

Evaluation of sensitivity of nested PCR

To evaluate the sensitivity of nested PCR, clinically isolated *H. pylori* was used. *H. pylori* was cultured for 7 days on modified TayerMartin media under microaerophilic condition[20]. An *H. pylori* suspension was made with normal saline, and the bacteria were directly counted using a Petroff-Hausser chamber (VWR Scientific Products, Willard, OH, USA) under microscope. After cell counting, the suspension was serially diluted with normal saline.

Statistical analysis

Data were analysed using the chi-square test, Fisher's exact test, Pearson's correlation coefficient, Spearman's correlation coefficient, and the score test for trend. A *P* value of less than 0.05 was considered as statistically significant.

RESULTS

Characteristics of the study population

one hundred and fourteen patients (69 males and 45 female) with gastroduodenal diseases were included in this study population. Their age ranged from 19 to 80 years with a mean age of 41±13.7 years. The gastroduodenal diseases consisted of 32 duodenal ulcers, 21 gastric ulcers, 38 gastritis & duodenal ulcers, and 23 gastritis.

The sensitivity of nested PCR

PCR assay was able to detect as few as 5 *H. pylori*/mL (Fig. 1).

The result of nested PCR and ^{13}C-urea breath test

A total of 82 (71.9%) out of 114 patients had positive ^{13}C-urea breath tests. Among these 82 patients, 21 (25.6%) were PCR positive in their saliva. No *H. pylori* was detected in the saliva of ^{13}C-urea breath test negative patients. The overall positive rate of *H. pylori* in saliva was 21 (18.4%) of 114. All patients with *H. pylori*-positive showed a high value (> 50 δ‰) of ^{13}C-urea breath test. The higher the ^{13}C-urea breath test value was, the higher was the detection rate of *H. pylori* in saliva (Fig. 2, P=0.001). ^{13}C-urea breath test values were not significantly correlated with age (r=0.099, P=0.335), sex (P=0.417), and diagnosis (Table 2, P=0.238). PCR assay in saliva was not significantly correlated with age (r=0.083, P=0.421), sex (P=0.154), and diagnosis (Table 2, P=0.686), either.

DISCUSSION

According to previous studies[11-17], the detection rates of *H. pylori* in saliva with PCR assays ranged from 0% to 75%. As the primers used in these studies targeted different genes and the prevalence of *H. pylori* was different according to geographic locations, the results are difficult to compare. In many studies[13-15,17], *H. pylori* was often present in the oral cavity and there was a significant correlation of *H. pylori* in the stomach and the oral cavity. However, several studies[16,21,22] suggested that *H. pylori* may be a transient oral microorganism, because *H. pylori* was frequently detected in gastric mucosa, but not in saliva or dental plaque. In another study[12], it was suggested that *H. pylori* may be normal oral microflora, because *H. pylori*

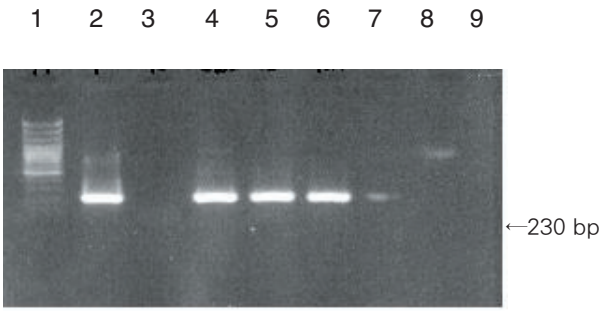

Fig. 1. Detection limits of nested polymerase chain reaction.
Lane 1, DNA ladder marker; lane 2, positive control; lane 3, negative control; lane 4, 100 *H. pylori*/mL; lane 5, 20 *H. pylori*/mL; lane 6, 10 *H. pylori*/mL; lane 7, 5 *H. pylori*/mL; lane 8, 2 *H. pylori*/mL; lane 9, 1 *H. pylori*/mL.

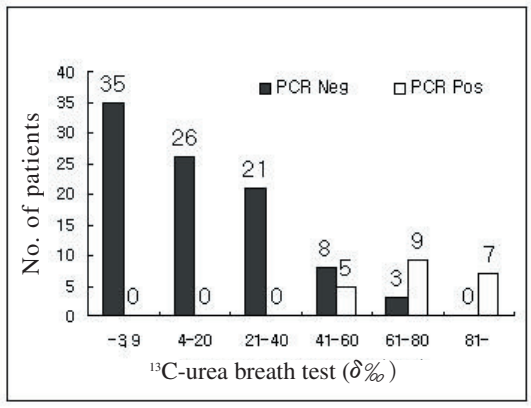

Fig. 2. Correlation between the value of ^{13}C-urea breath test and PCR assay(P=0.001). PCR Neg, PCR negative; PCR Pos, PCR positive.

was present in the oral cavity of patients, independently of gastric infection.

In this study, the detection rate of *H. pylori* in saliva was lower than in other studies[13-15,17], considering the high prevalence of *H. pylori* in Korea[23]. Some *H. pylori* might have escaped the PCR assay detection.

Followings are some speculations on the significance of the detection rate in saliva. In this study the sensitivity and specificity of PCR in various studies were compared and the highly specific and sensitive nested PCR assay was chosen. However, more sensitive techniques are needed. Jiang et al.[17] developed a PCR assay that was able to detect as few as 1 *H. pylori* in saliva. It has been also reported that collecting samples from the oral cavity at different time of the day also affect the detection rate. For example, saliva samples collected in the morning before teeth brushing gave a higher rate of detection of *H. pylori*[5]. In addition, *H. pylori* may not be uniformly distributed in the oral cavity[5,12,24]. In many studies[12-13,16], *H. pylori* was detected more frequently in dental plaque than in saliva. Further investigations on the correlation between *H. pylori* in the oral cavity and stomach, the presence of *H. pylori* in dental plaque, and repetitive saliva samples should be performed. Furthermore, it would be necessary to determine the DNA type of *H. pylori* isolates from both the oral cavity and stomach. DNA typing would provide more precise data on the relation between *H. pylori* in the oral cavity and the stomach. In addition, it is likely to play a major role in studying the mode of transmission.

In summary, the result of the present study showed that *H. pylori* is present in the saliva of patients with highly active gastric infections (〉50 δ‰). No *H. pylori* was detected in the saliva from patients without gastric infection. In patients with highly active gastric infection, *H. pylori* may be transmitted via saliva. The PCR assay of *H. pylori* in saliva is not useful for detecting gastric infection with *H. pylori* but may be a useful tool for the screening of highly infectious patients.

Acknowledgment

We thank Kuak Eun Young, M.T. for her technical assistance.

REFERENCES

1. Graham DY. *Campylobacter pylori and peptic ulcer disease. Gastroenterology 1989;96:615-25.*
2. Tayler DN, Blaser MJ. *The epidemiology of Helicobacter pylori infection. Epidemiol Rev 1991;13: 42-59.*
3. Forman D, Newell DG, Fullerton F, Yarnell JW, Stacey AR, Wald N, et al. *Association between infection with Helicobacter pylori and risk of gastric cancer: evidence from a prospective investigation. BMJ 1991;302:1302-5.*
4. Goodwin CS, Mendall MM, Northfield TC. *Helicobacter pylori infection. Lancet 1997;349:265-9.*
5. David RC. *Transmission and Epidemiology of Helicobacter pylori infection. Ame J Med 1996;100:12-8.*
6. Krajden S, Fuksa M, Anderson J, Kempston J, Boccia A,

Petrea C, Babida C, Karmali M, Penner JL. *Examination of human stomach biopsies, saliva, and dental plaque for Campylobacter pylori. J Clin Microbiol 1989;27:1397-8.*

7. Thomas E, Jiang C, Chi DS, Li C, Ferguson DA Jr. *The role of the oral cavity in Helicobacter pylori infection. Am J Gastroenterol 1997;92:2148-54.*
8. Ferguson DA Jr, Li C, Patel NR, Mayberry WR, Chi DS, Thomas E. *Isolation of Helicobacter pylori from saliva. J Clin Microbiol 1993;31:2802-4.*
9. Khandaker K, Palmer KR, Eastwood MA, Scott AC, Desai M, Owen RJ. *DNA fingerprints of Helicobacter pylori from mouth and antrum of patients with chronic ulcer dyspepsia. Lancet 1993;342:751.*
10. Luman W, Alkout AM, Blackwell CC, Weir DM, Palmer KR. *Helicobacter pylori in mouth-negative isolation from dental plaque and saliva. Eur J Gastroenterol Hepatol 1996;8:11-4.*
11. Bernander S, Dalen J, Gastrin B, Hedenborg L, Lamke LO, Ohrn R. *Absence of Helicobacter pylori in dental plaques in Helicobacter pylori positive dyspeptic patients. Eur J Clin Microbiol Infect Dis 1993;12:282-5.*
12. Song Q, Lange T, Spahr A, Adler G, Bode G. *Characteristic distribution pattern of Helicobacter pylori in dental plaque and saliva detected with nested PCR. J Med Microbiol 2000;49(4):349-53.*
13. Mapstone NP, Lynch DA, Lewis FA, Axon AT, Tompkins DS, Dixon MF, Quirke P. *Identification of Helicobacter pylori DNA in the mouths and stomachs of patients with gastritis using PCR. J Clin Pathol 1993;46(6):540-3.*
14. Li C, Musich PR, Ha T, Ferguson DA Jr, Patel NR, Chi DS, Thomas E. *High prevalence of Helicobacter pylori in saliva demonstrated by novel PCR assay. J Clin Pathol 1995;48:662-6.*
15. Li C, Ha T, Ferguson DA Jr, Chi DS, Zhao R, Patel NR, Krishnaswamy G, Thomas E. *A newly developed PCR assay of H. pylori in gastric biopsy, saliva, and feces. Evidence of high prevalence of H. pylori in saliva supports oral transmission. Dig Dis Sci 1996;41:2142-9.*
16. Oshowo A, Gillam D, Botha A, Tunio M, Holton J, Boulos P, Hobsley M. *Helicobacter pylori: the mouth, stomach, and gut axis. Ann Periodontol 1998;3(1): 276-80.*
17. Jiang C, Li C, Ha T, Ferguson DA Jr, Chi DS, Laffan JJ, Thomas E. *Identification of H. pylori in saliva by a nested PCR assay derived from a newly cloned DNA probe. Dig Dis Sci 1998;43(6):1211-8.*
18. Shimada T, Ogura K, Ota S, Terano A, Takahashi M, Hamada E, Omata M, Sumino S, Sassa R. *Identification of Helicobacter pylori in gastric specimens, gastric juice, saliva, and faeces of Japanese patients. Lancet 1994;343:1636-7.*
19. Mock T, Yatscoff R, Foster R, Hyun JH, Chung IS, Shim CS, Yacyshyn B. *Clinical validation of the Helikit: a ^{13}C urea breath test used for the diagnosis of Helicobacter pylori infection. Clin Biochem 1999;32:59-63.*
20. Paik IK, Kim YS, Shin WC, Lee JH. *Comparison of selective media for culture for Helicobacter pylori. Korean J Clin Microbiol 2001;4:11-5.*
21. Cammarota G, Tursi A, Montalto M, Papa A, Veneto G, Bernardi S, Boari A, Colizzi V, Fedeli G, Gasbarrini G. *Role of dental plaque in the transmission of Helicobacter pylori infection J Clin Gastroenterol 1996;22:174-7.*
22. Hardo PG, Tugnait A, Hassan F, Lynch DA, West AP, Mapstone NP, Quirke P, Chalmers DM, Kowolik MJ, Axon AT. *Helicobacter pylori infection and dental care. Gut 1995;37:44-6.*
23. Kim SY, Ahn JS, Ha YJ, Doh HJ, Jang MH, Chung SI, et al. *Serodiagnosis of Helicobacter pylori infection in Korean patients using enzyme-linked immunosorbent assay. J Immunoassay 1998;19:251-70.*
24. Nguyen AM, Engstrand L, Genta RM, Graham DY, el-Zaatari FA. *Detection of Helicobacter pylori in dental plaque by reverse transcription-polymerase chain reaction. J Clin Microbiol 1993;31:783-7.*

＝국문요약＝

타액에서 Nested Polymerase Chain Reaction을 이용한 *Helicobacter pylori* 검출

한태희, 백인기

인제대학교 상계백병원 임상병리과

배 경 : 이번 연구는 *H. pylori*에 감염된 환자의 타액에서 *H. pylori* 양성률과 *H. pylori* 감염 정도와 타액에서 *H. pylori*의 존재의 상관관계를 알아보자 하였다.

방 법 : *H. pylori* 의 위장감염은 ^{13}C-urea breath test로 확인하였고 타액에서 *H. pylori* 존재 여부는 시발체 EHC-U/EHC-L와 ET-5U/ET-5L를 이용하여 nested polymerase chain reaction으로 확인하였다.

결 과 : 중합효소연쇄반응은 타액 1mL 당 5개의 *H. pylori*를 검출할 수 있는 민감도를 보였다. 위십이장질환 환자 114명 중 ^{13}C-urea breath test 양성인 환자는 82명(71.9%) 이었으며 이들 82명 중 21명(25.6%)의 타액에서 중합효소연쇄반응을 이용하여 *H. pylori*를 검출할 수 있었고 이들은 모두 ^{13}C-urea breath test 값이 높았다(〉50 δ‰).

결 론 : *H. pylori*의 위장관 감염정도가 심한 환자의 경우 *H. pylori*가 타액을 통해 타인을 감염시킬 가능성이 있으며 타액에서 *H. pylori* 중합효소연쇄반응검사가 타인에게 *H. pylori* 감염을 일으킬 가능성이 높은 환자를 선별하는데 이용될 수 있을 것으로 판단되었다.