World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i12.3343

World J Gastroenterol 2014 March 28; 20(12): 3343-3349
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Detection of genotypic clarithromycin-resistant *Helicobacter pylori* by string tests

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Supported by Grants from National Science Council of Republic of China, No. NSC96-3111-P-042A-004-Y and No. NSC97-2314-B-037-047-MY3; and from Kaohsiung Medical University Hospital, No. KMUH97-7R32 and No. KMUH97-7G49
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Received: August 23, 2013 Revised: September 17, 2013
Accepted: October 19, 2013
Published online: March 28, 2014

Abstract

AIM: To evaluate the utility of the string test to detect genotypic clarithromycin-resistant *Helicobacter pylori* (*H. pylori*) by polymerase chain reaction (PCR)-restriction fragment length polymorphism.

METHODS: Patients undergoing endoscopic examinations were enrolled in the present study. String tests were done on the next day of endoscopy. Segments of 23S rRNA were amplified from DNA obtained from string tests. PCR-restriction fragment length polymorphism was accomplished by restriction enzymes BbsI and BsaI recognizing the mutation site A to G at 2143 or at 2142 of 23S rRNA domain V, respectively.

RESULTS: One hundred and thirty-four patients with *H. pylori* infection underwent string tests. To compare phenotypic resistance, 43 isolates were successfully cultured in 79 patients in whom 23S rRNA was successfully amplified. Of five patients with clarithromycin-resistant *H. pylori*, 23S rRNA of *H. pylori* isolates from four patients could be digested by BsaI. In 38 susceptible isolates, 23S rRNA of *H. pylori* isolates from 36 patients could not be digested by either BsaI or BbsI. The sensitivity and specificity of the string test to detect genotypic clarithromycin resistance were 66.7% and 97.3%, respectively. Positive and negative predictive values were 80% and 94.7%, respectively.

CONCLUSION: String test with molecular analysis is a less invasive method to detect genotypic resistance before treatment. Further large-scale investigations are necessary to confirm our results.

Key words: *Helicobacter pylori*; String test; Clarithromycin resistance; Polymerase chain reaction-restriction fragment length polymorphism

Core tip: According to region, antibiotic resistance is mostly detected by culture of endoscopic biopsy. The

study aimed to detect genotypic clarithromycin resistance by string tests. Amplified 23S rRNA from strings was digested by restriction enzymes to discriminate A2142G or A2143G mutations conferring clarithromycin resistance. Culture was also done to compare genotypic and phenotypic resistance. Sensitivity and specificity of the method were 66.7% and 97.3%, respectively. Positive and negative predictive values were 80% and 94.7%, respectively. Our study demonstrates that the string test, rather than endoscopic biopsy culture, could provide an option for molecular analysis in future.

Wu JY, Wang SSW, Lee YC, Yamaoka Y, Graham DY, Jan CM, Wang WM, Wu DC. Detection of genotypic clarithromycin-resistant *Helicobacter pylori* by string tests. *World J Gastroenterol* 2014; 20(12): 3343-3349 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i12/3343.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i12.3343

INTRODUCTION

Since *Helicobacter pylori* (*H. pylori*) was isolated in 1984[1], it has been widely believed to be a major cause of peptic ulcer, gastritis and mucosa-associated lymphoid tissue lymphoma (MALToma)[1-4]. Evidence from Mongolian gerbils and epidemiological studies suggests the carcinogenesis of *H. pylori* in gastric cancer[5,6]. It has been reported that increased production of reactive oxygen species and reactive nitrogen species by *H. pylori* leads to gastric inflammation and carcinogenesis[7]. Recent reports showed that eradication of *H. pylori* not only reduced the severity and recurrence of peptic ulcers and gastritis, but also diminished the chance of gastric cancer development[8-10]. Furthermore, remissions in MALToma were also proven after *H. pylori* was eradicated[11,12]. Thus, eradication of *H. pylori* is the uppermost important issue in gastric ulcer, duodenal ulcer, MALToma, atrophic gastritis, and gastric adenocarcinoma, as well as following gastric cancer resection[13].

Currently, several regimens for *H. pylori* eradication have been suggested, such as traditional triple, sequential, hybrid, and concomitant therapies[14-18]. However, one major cause of unsuccessful eradication is the presence of antimicrobial resistance[19]. In patients with metronidazole-resistant strains, 20% to 50% decreases in cure rates were noted with metronidazole-based combination regimens[20,21]. In clarithromycin-based triple therapy, treatment failure has been reported in more than 50% of patients with clarithromycin-resistant strains[22]. Therefore, early detection of antibiotic resistance could avoid treatment failure.

In regard to *H. pylori* antibiotic susceptibility tests (either agar dilution test or E-test), invasive endoscopic biopsy for culture of *H. pylori* isolates is necessary. However, technique-dependent culture procedures limit its clinical application for most general practices. Consequently, it is a practical issue to investigate more rapid and less invasive methods to detect antimicrobial resistance prior to eradication therapy.

The mechanisms of antibiotic resistance in *H. pylori* have been widely studied in previous studies. For clarithromycin, point mutations at "hot-spots" (A to G at 2142, 2143) in 23S rRNA domain V were proposed as the major mechanism of clarithromycin resistance of *H. pylori*[23]. With appropriate restriction enzymes (BbsI and BsaI), the mutations (A to G at 2142, 2143) were discriminated between susceptible and resistant strains by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[24].

It was reported that there were 10^7-10^8 organisms per milliliter of gastric juice[25]. Therefore, *H. pylori* in gastric juice could be detected by non-invasive tests such as bacterial culture, rapid urease test (RUT), and polymerase chain reaction (PCR) assay[26]. The string test was proven to be able to detect *H. pylori* with a high sensitivity and specificity[27]. Approximately 0.5 mL of gastric juice with *H. pylori* attached by every 10 cm of the string was reasonable for molecular biological analysis[28]. The aim of this study was to evaluate the string test to detect genotypic clarithromycin-resistant *H. pylori*.

MATERIALS AND METHODS

Patients undergoing endoscopic examinations at Kaohsiung Medical University Hospital were candidates in this study, which was approved by the local ethics committee. Patients who had taken antibiotics, bismuth salts, or proton pump inhibitors within one month, those who had ever received *H. pylori* eradication treatment or gastric surgery, and those who had a bleeding peptic ulcer, severe co-morbidity, or current pregnancy or lactation were all excluded. *H. pylori* infection was documented by the rapid urease test (RUT), culture, and histology in all enrolled patients. ^{13}C-UBT was also performed on the same day. Positive *H. pylori* infection was considered when either culture yield was positive or any two of the other three tests, including RUT, histology, and ^{13}C-UBT, were positive. The string test was carried out in patients with *H. pylori* infection on the following day.

The procedure of ^{13}C-UBT was modified from a previous protocol[29]. Briefly, the regimen consisted of ingestion of 100 mg ^{13}C-urea agent (manufactured by the National Nuclear Institute of Taiwan) following 100 mL of milk as a test meal. An overnight fast for at least 8 h was requested. After ingestion of the ^{13}C-urea agent, patients were asked to rinse their mouth out three times. Duplicate baseline breath samples were taken before ingestion, and 25 min after ingestion for the test. A mass spectrometry device was used to measure excess ^{13}C in breath samples. The result of ^{13}C-UBT more than 4/mL was defined as positive.

The string test (Entero-Test *H. pylori*, HDC Corporation, CA, United States) was used to detect *H. pylori* as previously described[27]. A 90-cm nylon string coiled inside a gelatin capsule was used. A free-end looped string

Table 1 Polymerase chain reaction primers and sequences used in this study

Primer name	Sequence	Annealing temperature	Size (bp)
cagA	5'-GAT AAC AGG CAA GCT TTTGAC G-3'	50 ℃	349
cagR	5'-CTG CAA AAG ATT GTT TGG CAG A-3'		
HP-K1	5'-CCA CAG CGA TGT GGT CTC AG-3'	54 ℃	425
HP-K2	5'-CTC CAT AAG AGC CAA AGC CC-3'		

The annealing temperature and the size of amplicons are also listed. The reaction conditions are detailed in Materials and Methods.

protrudes through a hole in the other end of the capsule. Before the capsule was swallowed, 10-20 cm of the free-end string was pulled out and its position was ensured by adhesion of a small piece of tape to the patient's cheek. It was swallowed with 300 mL of water after 8 h of fasting. One hour after swallowing, the string was retrieved in a swift motion to prevent gag reflex and discomfort. The capsule separated from the string during withdrawal and was passed into the stool; although minimal complications such as capsule retention can occur, these did not happen in our study fortunately. The withdrawn string yielded one to two mL of gastric juice and was placed in a sterile petri dish without any fluid to prevent dilution. The first 30 cm was discarded to preclude oral contamination. The string was then checked against the pH indicator. The segment of string, showing low pH as red color appearance, was most desirable. The string was then processed for extraction of DNA, PCR, cultured for *H. pylori* and CLO test.

Genomic DNA was extracted from the string as previously described[30]. To ensure the presence of *H. pylori* genes, the extracted DNA was first tested for the cagA gene by PCR, as the prevalence of the *cagA* gene of *H. pylori* in Taiwan is greater than 95%[31]. The PCR condition and the sequence of the primers were used as previously indicated (Table 1)[32]. Briefly, 20 μL of PCR mixture, containing 5 μL of extracted DNA, 200 μmol/L of (each) deoxynucleoside triphosphates (dNTPs), 0.4 μmol/L (each) primer, 1.5 mmol/L $MgCl_2$, and 1 U of Taq polymerase in PCR buffer [20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 0.2% glycerol], was held for 5 min at a denaturation temperature of 95 ℃, followed by 35 cycles of 30 s each at a denaturation temperature of 95 ℃, an annealing temperature of 50 ℃, and an elongation temperature of 72 ℃ and by 5 min at 72 ℃. The PCR products were analyzed by 1% agarose electrophoresis.

Among subjects with positive PCR amplification of the cagA gene, PCR-RFLP was done to elucidate the point mutations (A2142G and A2143G) of 23S rRNA, which were responsible for clarithromycin resistance of *H. pylori*[24,33]. PCR primers and conditions used to amplify the fragments of the peptidyl transferase region of the 23S rRNA are listed in Table 1. In brief, PCR amplification of DNA was performed in a final volume of 50 μL containing 100 ng of *H. pylori* genomic DNA, 75 mmol/L Tris-HCl (pH 8.8), 20 mmol/L $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of dNTPs, 1 mol/L of primers and 2 U of Taq DNA polymerase. The cycling program was 1 cycle at 95 ℃ for 5 min; 35 cycles of 95 ℃ for 30 s, 54 ℃ for 30 s, and 72 ℃ for 30 s, and a final elongation step at 72 ℃ for 10 min. Ten microliters of amplicon was incubated with restriction enzymes (24 h at 56 ℃ for BsaI and at 37 ℃ for BbsI) to discriminate wild type, A2142G mutant (BbsI restriction site), and A2143G mutant (BsaI restriction site)[24].

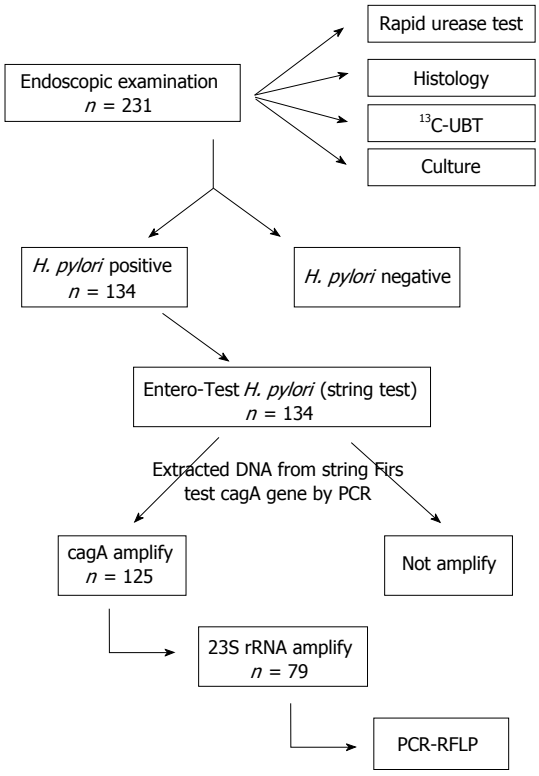

Figure 1 Trial profile. PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; *H. pylori*: *Helicobacter pylori*.

RESULTS

One hundred and thirty-four patients (58%) were proved to be infected with *H. pylori* by invasive and non-invasive methods as described in Materials and Methods. These patients underwent string tests on the next day of endoscopy. The trial profile is shown in Figure 1. To ensure that the retrieved string contained detectable amounts of *H. pylori*, PCR-based cagA gene detection was also done since the cagA gene is reported at a high detection rate in *H. pylori* from East Asia and Taiwan[31,34], and *cagA* genes were detected from 93.3% of strings (125/134), validating the usability of DNA (Figure 2). Eventually, segments of 23S rRNA were amplified in 79 of 125 patients with positive gene amplifications from strings. To elucidate the point mutations (A2142G and A2143G) of 23S rRNA which are the main mechanism of clarithromycin resistance of *H. pylori*, restriction enzymes (BsaI and BbsI) were applied. Seventeen amplicons possess BsaI-recognizable restriction site (*i.e.*, A2143G mutant),

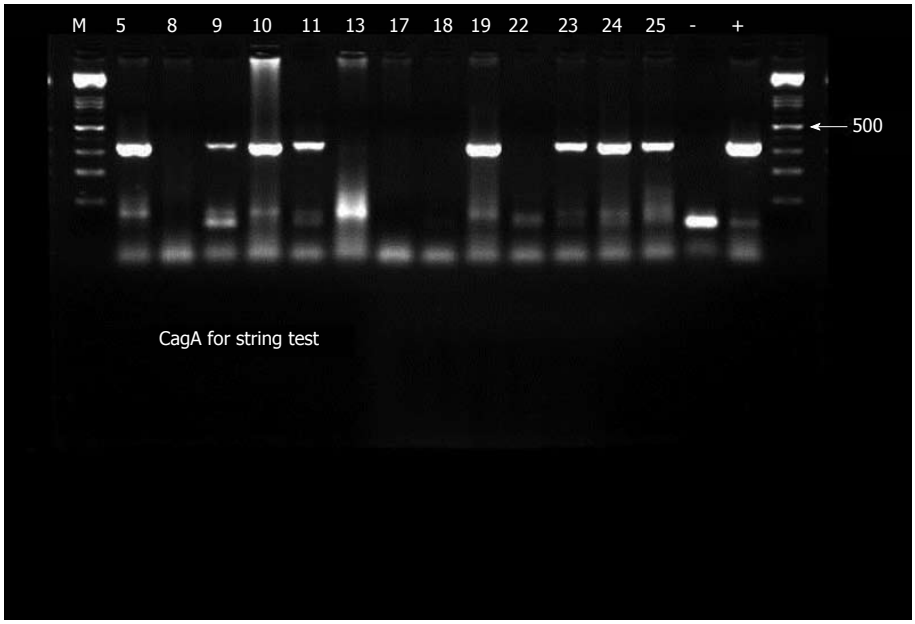

Figure 2 Polymerase chain reaction amplification of the cagA gene. The numbers on the top row indicate the patient numbers. M: Marker; -: Negative control; +: Positive control.

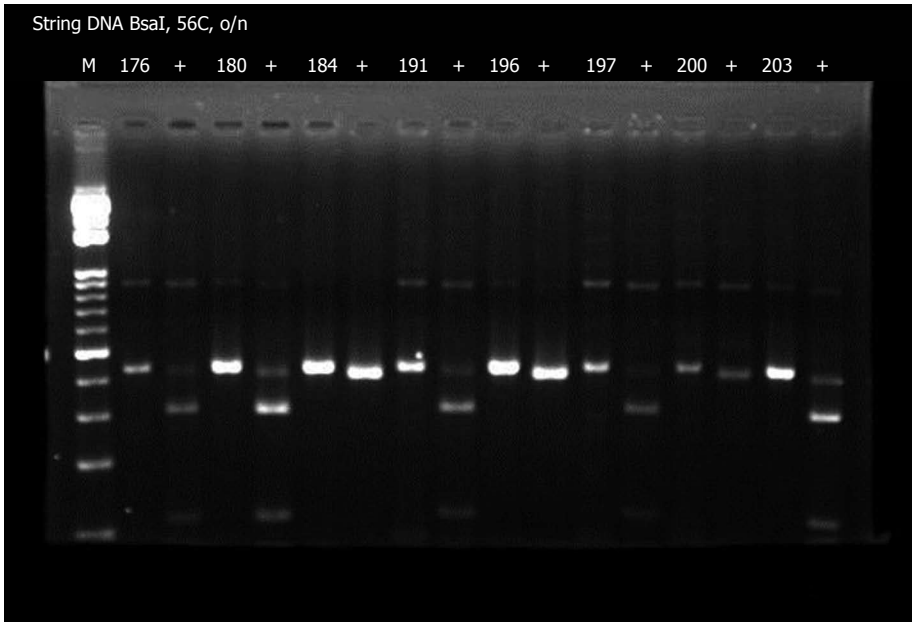

Figure 3 Polymerase chain reaction-restriction fragment length polymorphism for detection of clarithromycin resistance. The numbers on the top row indicate the patient numbers. M: Marker; +: Amplicons digested by BsaI. Clarithromycin-resistant *Helicobacter pylori* isolates from five patients (patient Nos. 176, 180, 191, 197, and 203) showed positive reactions.

whereas none of the 79 amplicons had the restriction site for BbsI (A2142G mutant) (Figure 3).

Culture from string is affected by several factors such as transportation, storage, and the contamination from oral flora, and is therefore more difficult than biopsied tissues. Among 79 patients who underwent string-based PCR-RFLP, *H. pylori* was successfully cultured in 43 patients. Among 43 *H. pylori* isolates, five (11.6%) were phenotypic-resistant to clarithromycin by E-tests and four were genotypic-resistant by BsaI digestion (*i.e.*, A2143G mutant). Otherwise, 36 isolates were not digested by either BsaI or BbsI in 38 phenotypically susceptible *H. pylori* (Table 2). Therefore, the sensitivity and specificity of string-based PCR-RFLP to detect genotypic clarithromycin resistance of *H. pylori* were 66.7% and 97.3%, respectively. Positive and negative predictive values were 80% and 94.7%, respectively.

DISCUSSION

Since *H. pylori* eradication failure is caused mainly by antimicrobial resistance, detection of resistance prevalence

Table 2 Result of polymerase chain reaction-restriction fragment length polymorphism from the string test

		String PCR-RFLP	
		Sensitive	Resistant
E-test	Sensitive	36	2
	Resistant	1	4

Five were resistant to clarithromycin as confirmed by E-tests in 43 *Helicobacter pylori* isolates. Among these 43 patients undergoing the string test, four 23S rDNA amplicons from strings were digested by BsaI, indicating a mutation site A to G at 2142 of 23S rRNA. PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism.

is critical for choice of treatment strategy[22]. Among antibiotics for *H. pylori* eradication, clarithromycin resistance plays an important role in eradication failure when clarithromycin-based triple therapy is used[19,35]. For susceptibility testing, *H. pylori* culture is always mandatory but not practical for most general practices due to technical difficulty. Therefore, empirical therapy was suggested by regional resistance prevalence[13]. The current study aimed to develop a less invasive method for detection of genotypic clarithromycin resistance before "test-and-treat"[36].

Clarithromycin resistance of *H. pylori* caused by single point mutations within 23S rRNA has been reported[37]. A to G mutations at positions 2143 and 2144 of 23S rRNA were proposed as one of the major causes of clarithromycin resistance[38]. Although other mutations (A2142C, A2143C, A2115G, G2141A, and A2142T) have also been reported to be associated with resistance to clarithromycin, studies from East Asian countries have shown that more than 90% of the mutant strains had the A2143G mutation instead of the A2142C mutation[39]. Another study in China showed that gene mutation rates of A2142C, A2142G, and A2143G in the 23S rRNA gene were 1.5% (1/65), 6.2% (4/65), and 84.6% (55/65), respectively[40]. Clarithromycin-resistant *H. pylori* with the A2143G mutation possesses a recognizable sequence ($_{2143}$GAGACC$_{2148}$) by restriction enzyme *Bsa*I, whereas the sequence of resistant strains with the A2142G mutation is recognized by *Bbs*I. Therefore, appropriate restriction enzymes (*Bbs*I and *Bsa*I) can be used to discriminate susceptible and resistant strains at "hot-spot" mutations (A2142G or A2143G)[24].

Because bacterial culture is not always satisfactory, the string test was used to detect *H. pylori* as described in a previous report[27]. This was a gastric juice-based PCR to detect the bacteria and tissue obtained by the string. According to the previous result, string test-based PCR for the detection of *H. pylori* was accurate, convenient, and well tolerated by patients. Besides the detection of *H. pylori*, it also carried approximately 0.5 mL of gastric juice containing PCR-detectable yields of bacteria absorbed by every 10 cm of the string. Therefore, the utility and efficiency of string tests for detection of *H. pylori* have been well established in several studies[41,42].

High cagA detection rate (93.3%, 125 out of 134 patients) merited the usability of DNA from string for providing further molecular analysis in the present study. In positive cases, 23S rRNA was successfully amplified in 79 cases whereas *H. pylori* was successfully cultured from strings in 43 patients. Among 43 isolates, five (11.6%) were resistant to clarithromycin with similar antibiotic resistance prevalence in the same region[43]. 23S rRNA from string DNA of four patients was digested by *Bsa*I among the five patients with clarithromycin-resistant *H. pylori*. DNA from 36 strings was not digested by either *Bsa*I or *Bbs*I in 38 patients with clarithromycin-susceptible *H. pylori*. In further analysis by bacterial DNA from culture, five resistant isolates possessed the A2143G mutation, which was compatible with previous reports that more than 90% of the resistant strains had the A2143G mutation in Asia[44].

In conclusion, the sensitivity and specificity of string-based PCR-RFLP for detection of genotypic resistance of clarithromycin were 66.7% and 97.3%, respectively, in the present study. Positive and negative predictive values were 80% and 94.7%, respectively. Our study provided a possible option for less invasive genotypic analysis of clarithromycin resistance rather than culture of endoscopic biopsy. However, further large-scale investigations are necessary to confirm our results.

COMMENTS

Background

One major cause of unsuccessful *Helicobacter pylori* (*H. pylori*) eradication is the presence of clarithromycin resistance. Phenotypic resistance always requires susceptibility tests by culture. Evaluation of genotypic clarithromycin resistance is considered to have an important role for successful treatment. Therefore, the study was designed to validate the string test to detect genotypic clarithromycin-resistant *H. pylori*.

Research frontiers

By appropriate molecular analysis such as polymerase chain reaction-restriction fragment length polymorphism, the string test could be a clinically useful tool to detect genotypic clarithromycin-resistant *H. pylori*.

Innovations and breakthroughs

This paper is the first study to detect genotypic clarithromycin resistance by the string test. Several papers have been published to detect *H. pylori* by the string test, but none of them have discussed antimicrobial resistance. The innovations of our study provide an option for less invasive genotypic analysis such as antibiotic resistance surveillance.

Applications

By the methods in the study, genetic analysis of *H. pylori* can be achieved by the string test rather than technical-dependent culture of invasive endoscopic biopsy.

Terminology

Genotypic resistance: organisms possessing well-known genetic mutations leading to antimicrobial resistance are considered genotypically resistant. Phenotypic resistance: resistance of organisms to antibiotics as revealed by antibiotic susceptibility tests.

Peer review

This study presented a new method to examine clarithromycin-resistant *H. pylori*, which is most important for successful treatment. Its topic, aim and methods are very interesting and really attractive. Presentation and composition of the article are also sound. This study provides many possibilities for the string test in the genetic molecular analysis in future.

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ISSN 1007-9327

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