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Molecular characterization of *Helicobacter pylori* strains isolated from cynomolgus monkeys (*M. fascicularis*)[☆]

Sonia Q. Doi^a, Tara Kimbason^a, James Reindel^b, and Andre Dubois^{a,*}

^a Department of Medicine, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Room A3075, Bethesda, MD 20814, USA

^b World Wide Safety Sciences, Pfizer Global Research and Development, Ann Arbor, MI, USA

Abstract

We recently reported the occurrence of natural infection with *H. pylori* in a group of cynomolgus monkeys with chronic active gastritis and gastric erosions. The goal of the present study was to characterize and to compare strains isolated from animals originating from two different geographical areas. Gross and microscopic pathology determined at the time of necropsy was similar in all animals. *H. pylori* were isolated from specimens harvested in five monkeys (four from Vietnam and one from the Philippines) with gastritis. Isolates from monkeys bred in Vietnam had a similar DNA fingerprint pattern, which was distinct from that of isolates from a monkey bred in the Philippines. All strains were of the *sla vacA* subtype, but all the ‘Vietnamese’ strains were *cagA*⁺ and all but one were *iceA1* whereas the ‘Philippino’ strains were *cagA*[−] and *iceA2*. The sequences of the 16S rRNA of the Vietnamese and Philippino strains shared 98% homology and both clustered with *H. pylori* sequences present in the NCBI database. In conclusion, cynomolgus monkeys can be naturally colonized by *H. pylori*, and the strains isolated from these animals appear to vary according to the geographical origin, thus indicating probable infection prior to importation. Since some of the cynomolgus monkeys developed antral erosions during natural infection, we propose that this animal model may be used to investigate the role of *H. pylori* in ulcerogenesis.

Keywords

Nonhuman primate; Cynomolgus monkey; Gastritis; Gastric erosions; 16S ribosomal RNA; *Helicobacter pylori*; DNA fingerprinting; DNA sequencing; Genotyping; Stomach; Animal model

1. Introduction

Helicobacter pylori persistently colonizes the stomach of over half of humans worldwide, is consistently associated with chronic active gastritis (Dubois et al., 2000) and is as a major risk factor for peptic ulcer disease (Anon, 1994) and distal gastric cancer (Wong et al., 2004). The natural reservoir for *H. pylori* is believed to be limited to humans, certain macaques (*M. mulatta*, *M. fascicularis*, and *M. nemestrinae*) (Dubois et al., 1995; Reindel et al., 1999; Suerbaum et al., 2002), and cats (Handt et al., 1994).

[☆]The experiments reported herein were conducted according to the principles set forth in the “Guide for the Care and Use of Laboratory Animals”, Institute of Laboratory Animal Resources, National Research Council, HHS/NIH Publ. no. 85–23. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense, the Uniformed Services University of the Health Sciences.

*Corresponding author. Tel.: +1 301 295 3607; fax: +1 301 295 3557. E-mail address: adubois@usuhs.mil (A. Dubois).

The high prevalence of *H. pylori* and its potential pathogenicity stimulated the development of specific and sensitive methods for the detection of this microorganism. Culture of *H. pylori*, complemented by polymerase chain reaction (PCR) amplification has been a useful identification tool. A PCR-based DNA fingerprinting method termed random amplified polymorphic DNA (RAPD) allows tracing of an infecting strain in a given individual or among multiple subjects (Akopyants et al., 1992). Additional characterization of *H. pylori* strains by genotyping has demonstrated an association between some of the virulence genes and pathogenicity (Cover et al., 1997). However, using PCR for genotyping may be difficult due to the extensive polymorphism of many *H. pylori* genes and the absence of specific virulence genes in some strains. The sequencing of the 16S rRNA gene offers an alternative step in the sensitive detection and precise identification of *H. pylori*. In addition, the 16S rRNA gene sequence was used as a tool for detection and identification of *H. pylori* and other *Helicobacter* organisms especially from animal sources (Ho et al., 1991; Drazek et al., 1994; Fox et al., 1998).

The goal of the present study was to characterize and to compare strains isolated from animals with gastritis and mucosal erosions and originating from two different geographical areas. We genotyped *H. pylori* strains recovered from naturally-infected cynomolgus monkeys originating in Vietnam and the Philippines using RAPD and PCR techniques. Two 16S rRNA sequences representing one strain from the Philippines and one from Vietnam were decoded and aligned to determine the degree of identity between them. Each 16S rRNA sequence was also aligned to other sequences with the highest degree of homology available in the National Center for Biotechnology Information (NCBI) nucleotide database to identify a clustering group.

2. Methods

2.1. *H. pylori* strains

Isolates used in the present study were cultured from 5 colony-bred, 2.5–3.5 years old, cynomolgus monkeys (*Macaca fascicularis*) that had been obtained from Hazelton Research Products Inc., and kept in individual cages at an AALAC-accredited laboratory. One of the animals (no. 1200), originated from the Philippines and was among the 63 monkeys that were the subject of a previous report (Reindel et al., 1999). The other four animals (nos. 1205, 1207, 1224 and 1225) were from Vietnam and had been found to present with gastritis at necropsy. All animals were kept in individual cages in our facility at all times.

The health status of the monkeys was monitored routinely as described and none of the animals displayed signs of disease (Reindel et al., 1999). Following euthanasia, all animals were subjected to necropsy and specimens from the antro-pyloric mucosa were collected, cultured, isolated, and stored as described (Dubois et al., 1999). The remainder of each stomach was fixed in 10% neutral buffered formalin and processed as described (Reindel et al., 1999).

2.2. DNA extraction

DNA was extracted from each isolate collected in TE buffer as published (Atherton et al., 1997).

2.3. Random amplification of polymorphic DNA (RAPD)

DNA fingerprinting by RAPD was performed to compare the isolates using a set of five different 10-mer primers as previously published (Akopyants et al., 1992).

2.4. Genotyping

DNA from each *H. pylori* isolate was subjected to typing for *vacA* and *cagA* genes, using standard PCR amplification and specific primer sets for each target gene (Atherton et al., 1997; Cover et al., 1994).

2.5. 16S rRNA amplification and sequencing

The 16S rRNA gene of isolates collected from the Philippino monkey (1200) and the Vietnamese monkey (1205) were PCR-amplified with primers according to a previously published procedure (Eckloff et al., 1994). The ethidium bromide stained-DNA band visualized by UV transillumination was cut out from the agarose gel and purified using a GlasPac/GS QuicKit (National Scientific Supply Company Inc., San Rafael, CA). The sequence of the purified 16S rRNA gene was determined using sets of overlapping primers (Eckloff et al., 1994), a dye terminator reagent (PE Applied Biosystems, Foster City, CA) and an automated DNA sequencer (PE Applied Biosystems, Foster City, CA). The Gene Jockey Sequence Processor software (Biosoft, Cambridge, UK) was used to align the overlapping sequenced segments of the 16S rRNA gene. For the other three isolates, approximately 400 bases downstream from the 3' and upstream from the 5' ends were sequenced for comparison.

2.6. Alignment of the two decoded 16S rRNA sequences to each other and to other NCBI sequences

Homology between the two sequences (isolates from monkeys 1200 and 1205) and between each sequence and those currently available in the NCBI database was assessed using the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, NIH, <http://www.ncbi.nlm.nih.gov/BLAST>) score system. The scores assigned in a BLAST search have a well-defined statistical interpretation, making real matches easier to distinguish from random background hits. This scoring system also takes into account the total number of bases in the sequence analyzed. Therefore, scores from different BLAST searches are not comparable if the query sequences have different number of nucleotides.

3. Results

3.1. Microbiological characterization of *H. pylori* isolated from cynomolgus monkeys

Gram negative, curved, spiral or 'gull-wing'-shaped bacteria grew in cultures from all 5 animals within 7–10 days, forming pinhead-sized colonies with 'water-spray' morphology. Bacteria measured 3–6 µm in length and were positive for urease, oxidase, and catalase activity. A total of 19 single colony isolates [from the Philippino monkey, no. 1200 ($n = 2$), and from Vietnamese animals, no. 1205 ($n = 5$), 1207 ($n = 4$), 1224 ($n = 4$) and 1225 ($n = 4$)] were collected and further characterized at the molecular level.

Histologic lesions consisting of erosions and variable degrees of mucosal inflammation were as previously described (Reindel et al., 1999). No differences were noted between the monkeys from Vietnam and the Philippines.

3.2. DNA fingerprinting

RAPD fingerprinting analysis was performed to study the genomic diversity among the 19 isolates. The patterns of the two isolates from animal 1200 were identical in regard to all five primers used for RAPD. In contrast, the pattern of 17 isolates collected from the other four animals, although similar to each other, were quite different from the pattern found in the isolates of monkey 1200 (Fig. 1). Therefore, we identified two groups of isolates according to RAPD pattern variations: one from monkeys bred in Vietnam and the other from the monkey bred in the Philippines.

3.3. Genotyping

PCR amplification of *vacA* and *cagA* genes was performed to examine the pattern of expression of these genes among the isolates. The two isolates from monkey 1200 were of the *vacA-sla* genotype, and were negative for *cagA* and for the *m1/m2 vacA* subtype. All single colony isolates from the other four animals were *cagA* positive, of the *sla/m2 vacA* subtype. These results were also consistent with divergence between the two groups of isolates.

3.4. Sequencing and comparison between 16S rRNA sequences of two strains with divergent genetic patterns

The 16S rRNA sequences of strain 1200 and 1205 were decoded and registered in the GenBank nucleotide database under accession number AY155586 and AY155587, respectively. These strains were distinct from one another based on RAPD pattern and type of virulence genes.

Alignment of the 16S rRNA sequence of strain from animal 1200 (1448 bases) against that of isolate from monkey 1205 (1340 bases) showed an identity of 98%. As mismatches between these 16S rRNA sequences were found to be clustered toward the 5' and 3' ends, we sequenced both ends of the 16S rRNA (~400 bases at each end) of representative isolates from three other cynomolgus monkeys (1207, 1224, and 1225). There was a complete identity between these three sequences and that of strain 1205.

3.5. Comparative alignment with other *H. pylori* 16S rRNA sequences

A BLAST search for the 16S rRNA sequence of strain 1205 listed 34 sequences sharing $\geq 98\%$ homology with the query (Table 1). These strains were all *H. pylori*, and strain 1200 was listed as number 26 in descending order of identity, further supporting that although both strains 1200 and 1205 clustered with those of *H. pylori*, they were quite distinct from one another. Interestingly, the highest identity score for strain 1205 identified *H. pylori* strains isolated from a Rhesus monkey originating in India (GenBank accession no. U00679) previously studied in our laboratory (Drazek et al., 1994) and from a *M. nemestrinae* (GenBank accession no. AF348617) originally named *H. nemestrinae* and now recognized to be *H. pylori* (Suerbaum et al., 2002). In contrast, the highest identity score for strain 1200 listed *H. pylori* of human origin and these two isolates from macaques showed a lower degree of identity.

4. Discussion

In the present study, we characterized *H. pylori* isolates collected from naturally-infected cynomolgus monkeys originating from two different countries (Vietnam and the Philippines). Both morphological and enzymatic properties were consistent with *H. pylori* for all the isolates studied, but strains isolated from animals from Vietnam and from the Philippines differed at the molecular level. Geographical variations of *H. pylori* strains also have been reported in humans (Falush et al., 2003).

First, the pattern of RAPD fingerprinting analyzed by four different primers was similar among the isolates from the Philippines, but quite distinct from that of the monkeys from Vietnam. The strains also differed in that only strains from Vietnam were positive for the cytotoxin-associated gene (*cagA*). Although *cagA*⁺ strains are more frequent in patients with peptic ulcer disease (Censini et al., 1996), the nature and degree of gastric inflammation or erosions was similar in cynomolgus monkeys from these two countries. This lack of correlation between pathological findings and expression of the *cagA H. pylori* virulence gene suggests that, as has been reported in humans (Monack et al., 2004), the macaque host response may overcome bacterial virulence factors. Finally, alignment of the 16S rRNA sequences of strains 1200 and 1205 revealed a number of mismatches (approximately 2%), and the lists of sequences in

descending order of identity relative to each individual strain (1200 and 1205) were quite different.

H. pylori was first identified as the *Helicobacter* species that infected primarily humans while other *Helicobacter* species (*H. felis*, *H. rappini*, etc) infected animals. However, among the large number of *H. pylori* strains recently identified, several were found in macaques (Dubois et al., 1995; Handt et al., 1994; Reindel et al., 1999). Some of those strains were first classified as a different species (e.g. *H. nemestrinae*) and later recognized to be *H. pylori* (Suerbaum et al., 2002). It is important to note that the revised GenBank description of the strain *H. nemestrinae* under 'source' and 'organism' reflects the correction, although the former name of *H. nemestrinae* associated with the accession number still remains. One of the major elements used for identification was the 16S rRNA sequence. Finally, two of the strains that best matched strain 1205 were published in GenBank as *Helicobacter* sp. 'liver' (AF142583 and AF142585) although the phylogenetic tree subsequently demonstrated that they were in fact *H. pylori* (Avenaud et al., 2000).

The source of *H. pylori* isolated from macaques has not been established. In our previous study (Drazek et al., 1994), the close match between the 16S rRNA sequence of a rhesus monkey isolate and that of isolates from humans led us to conclude that cross-transmission had occurred at some point in the past. We propose that cross-transmission also occurred in *M. fascicularis*. Humans, other macaques, or the environment would be considered possible sources of infection. Animal handling practices of non-human primates in the United States make human-to-monkey transmission of these bacteria highly unlikely in this series. The potential in-house spread of infection amongst cynomolgus monkeys was not determined, but is possible. Natural transmission at our facility was unlikely as animals were housed in individual cages upon arrival, cages were cleaned daily, and individual food dishes were not shared and were regularly sanitized. However, we cannot exclude that accidental transmission occurred during administration of dosing suspensions via the use of gastric gavage tubes and oral speculae. Although common gavage tubes were used for dosing monkeys in each individual dose group and transfer of saliva and gastric contents were therefore possible within each treatment group, not all animals within specific dose groups had identifiable infections or gastric lesions.

The present findings have relevance for toxicity testing in non-human primates since cynomolgus monkeys now are routinely used for safety evaluation of drug candidates. In such studies, gastritis and erosions induced by *H. pylori* can be problematic and confound diagnosis of potential compound-related toxicity to the gastric mucosa. Recognition of *H. pylori* infection and associated gastritis is important to prevent inadvertent ascription of gastric inflammation as a compound effect. Furthermore, once infection is identified, steps can be taken to eliminate infection and associated gastritis in the colony (Dubois et al., 1998).

In summary, cynomolgus monkeys can be naturally colonized by *H. pylori*, and the strains isolated from these animals appear to vary according to the geographical origin. We conclude that cross-infection between macaques and humans may have occurred at some point either during breeding or in previous generations of animals.

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Fig. 1.

Representative DNA fingerprinting (RAPD) of *H. pylori* isolates. Animal 1200 originated from the Philippines, and the other four animals originated from Vietnam. Note that the two isolates from animal 1200 have identical RAPD. The pattern of the isolates from the other four animals, although similar to each other, were quite different from that of the isolates from monkey 1200.

Table 1

Results from a BLAST search using 16S rRNA sequence of strain 1205 (AY155587) as a query

	GenBank accession number	Blast score (bits)
1	U00679	2569
2	AF348617 ^a	2569
3	AF363064 ^a	2565
4	AF512997	2553
5	Z25742	2553
6	U01331	2553
7	U01328	2553
8	U01332	2545
9	AF535196	2543
10	AY062898	2537
11	U01330	2537
12	AF535198	2535
13	AF535197	2535
14	AY366421	2531
15	AY366424	2529
16	AF302106	2529
17	AY366422	2524
18	Z25741	2522
19	AF361935	2518
20	U01329	2514
21	AY062899	2506
22	AE001556	2506
23	AF535194	2504
24	AE000644	2504
25	AE000620	2502
26	AY155586 ^b	2494
27	AF535195	2488
28	AY366423	2486
29	AY364440	2486
30	M88157	2486
31	AY364438	2470
32	AY456638	2462
33	U08906	2456
34	AY364439	2418

Among the first 34 genes listed, only sequences of *H. pylori* 16S rRNA were identified.

^aSequences deposited in GenBank as 16S rRNA of *H. nemestrinae*, now *H. pylori* (Suerbaum et al., 2002).

^bStrain 1200.