Research Article

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Impact of neutrophil-activating protein conservation on diagnostic tests and vaccine design

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Abstract

BACKGROUND: The neutrophil activating protein (NAP) is a highly immunogenic and virulence factor of *Helicobacter pylori*, presenting inflammatory and immunomodulatory activity. Consequently, NAP has been explored as a diagnostic and therapeutic target. However, when evaluating a target protein to design diagnostic methods or vaccines, it is critical to determine the protein conservation among the bacterial population, as well the impact of alterations of amino acid residues on the protein antigenic profile.

RESULTS: In the present work, NAP conservation and theoretical antigenicity were determined among 51 sequences from *H. pylori* isolated from patients worldwide. A high NAP conservation (83%) was observed, where 17 amino acid residues, among the 144 residues of the protein, were polymorphic. Alterations at these polymorphic sites had a theoretically low impact on predicted antigenicity, where only 5 NAPs out of 51 NAPs presented a slightly different antigenic profile in relation to the consensus sequence. According to that, it was possible to recognize in western blotting 93% of NAP from different bacteria ($n = 15$) using polyclonal antibodies developed against a specific NAP.

CONCLUSIONS: It was predicted that when working with polyclonal antibodies or large NAP fragments for diagnostic and vaccine design, slight variation in protein sequence will have a minimal impact on NAP recognition. However, if a NAP monoclonal antibody or small NAP epitopes are considered, it is critical to select the most conserved and antigenic NAP regions, to maximize the coverage of NAP variants.
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Keywords: *Helicobacter pylori*; NAP; immunogenic; genetic variability; neutrophil-activating protein; immunovariability

INTRODUCTION

Helicobacter pylori is the main etiological agent of diverse gastric diseases, such as chronic gastritis and peptic and duodenal ulcer disease, and represents a relevant risk factor of gastric cancer and mucosa-associated lymphoid tissue lymphoma.[1] The neutrophil-activating protein (NAP) has been posited as a critical *H. pylori* virulence factor associated with the host chronic inflammatory status that leads to disease development.[2]

NAP is a small polypeptide (with 144 amino acid residues, 17 kDa), organized in a ball-shaped dodecamer structure.[3,4] NAP is structurally similar to the DNA protecting proteins described in Enterobacteriaceae, belonging to the stress protein class induced during long periods of nutrient deprivation.[3] NAP activity was originally associated with iron binding, since the hollow central part of the dodecamer can bind up to 500 atoms of iron.[3,4] Based on NAP sequence similarities and due to its iron high-binding capacity, NAP was originally classified as a bacterial ferritin. The effect of its iron-binding ability in pathogenesis is not clear. While some data indicate that NAP is not regulated by iron depletion or overload,[5] other identify diverse genes as cytotoxin associated A (*cagA*) gene and vacuolating cytotoxin A (*vacA*) gene and *nap* as regulated by iron.[6] The iron-binding activity of the protein may also be associated with NAP apparent antioxidant activity, preventing bacterial oxidative damage, since it is up-expressed in situations associated with an apparent compensatory response of bacteria to loss of resistance factors to oxidative stress such as antioxidant enzymes.[7,8] Also, *H. pylori* with *nap* negative mutants presents lower survival rates than the wild-type bacteria upon exposure to oxidative stress conditions.[6,9,10]

The NAP major activity, and current name, is associated with its capacity in attracting and activating neutrophils, promoting the adhesion of these immune cells to endothelium and the production of reactive oxygen intermediates and chemokines. The large infiltrations of neutrophils and monocytes are a characteristic of

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H. pylori infection. NAP attracts and activates diverse other types of leukocytes, such as monocytes and mast cells, while polarizing the antigen-specific T-cell responses towards T-helper type 1 phenotype (Th1).[2,11] NAP may also promote the adhesion of bacteria to the gastric mucin,[2] contributing, therefore, to the reduction of bacterial wash-out from the stomach. NAP proinflammatory activity may also promote the release of nutrients from the inflamed tissue for bacterial growth.[2,12,13]

The *nap* gene is considered a relevant virulence factor for the *H. pylori* infection process, and especially for the development of gastric diseases. Consequently, this gene is present and expressed in most strains. For example, the following four studies confirmed the *nap* presence, by gene sequencing, in 100% of the strains: four strains isolated from Russian patients[14]; and 21 strains,[15] 20 strains[16] and 12 strains[17] isolated from USA patients. In a study covering a higher population dimension (151 strains isolated from Chinese patients), 148 out of 151 presented the gene.[18] Considering gene expression, while Evans *et al.*[15] observed that 100% of the strains expressed the *nap* gene, Tang *et al.*[18] observed that 141 (95.9%) strains expressed the protein out of 147 that presented the gene.

Such as other virulence factors from *H. pylori*, e.g. CagA and VacA and Ure, NAP is an immunodominant antigen, as infected patients frequently present anti-NAP antibodies.[10,19] Consequently, NAP has been proposed for diagnostic kits, as a vaccine candidate alone or in mixtures of other *H. pylori* antigens, and as a therapeutic target to counteract *H. pylori*-induced inflammation.[13,20-22] The NAP immunostimulatory and immunomodulatory function has also been proposed, e.g. as an adjuvant for immunotherapies to enhance the immunogenicity of poor immunogens, as an immunomodulator, e.g. for treatment of allergic diseases.[23-25]

Due to the wide potential applications of NAP, it is critical to evaluate NAP conservation among the *H. pylori* population and the impact of variations of the protein sequence on the antigenic profile. The *nap* gene presents a high genetic diversity. For example, in a study considering 51 *nap* gene sequences retrieved from *H. pylori* worldwide, a high genetic variability was observed, with 88% of the *nap* sequences representing different alleles.[23] In the work presented here, the corresponding protein conservation, among the 51 *nap* gene sequences described in Calado,[23] was evaluated. Also studied was the impact of local variations of these 51 sequences on predicted antigenicity, and how polyclonal antibodies generated towards a specific NAP sequence recognize NAP from 15 other strains. The study will, therefore, contribute to a more comprehensive knowledge of *H. pylori* NAP conservation and antigenic variability and consequently of NAP as a target for disease diagnosis and vaccination.

MATERIALS AND METHODS

NAP conservation

Sequences of the *nap* gene from *H. pylori* were obtained from the EMBL database – EBI European Nucleotide Archive (http://www.ebi.ac.uk). These sequences were from bacteria isolated from patients of different world locations, including: 14 European, 15 Asian, 15 American, 4 Australian and 3 African (Table 1). The NAP sequence alignments were performed with MEGA4[26] using ClustalW method[27] of nucleotide sequences or homologous proteins, respectively. Regions with gaps or missing data were not considered, 432 positions having been considered from a total set of 438 base pairs. The coding region was defined by the universal genetic code.

NAP antigenicity

The consensus sequence of NAP was obtained by alignment of protein sequences using the previously mentioned method. The prediction of antigenicity of the whole consensus NAP and NAP variants was performed using the following methods (based on Lasergene tools of the ProteanTM® software V8 DNASTAR):

- -Jameson–Wolf method, which predicts potential antigenic determinants by combining three algorithms that calculate the antigenicity of the sequence: hydrophilicity (Hoop–Woods), surface probability (Emini) and flexibility of the protein backbone (Karplus–Schultz).[28] In short, the Jameson–Wolf 'antigenic index' results in a weighted combination of these three measures and may be useful for determining linear epitopes.
- Rothbard–Taylor method, which locates 80% of potential T antigenic determinants, by comparing to known common sequence motifs.[29]
- Sette MHC Motifs method, which predicts Th epitopes by sequence pattern analysis.[30]
- Amphipathicity prediction method (AMPHI), using the Margalit and Berzofsky algorithm, to predict antigenic Th sites[31] from the primary sequence, based on the identification of amphipathic helices. The results could be represented by a hydrophobicity graph, an AMPHI region graph, an alpha-helix graph and/or an AMPHI intensity graph.

Development of recombinant NAP and generation of the corresponding polyclonal antibodies

H. pylori growth and cell extract

In total, 15 strains of *H. pylori* (Table 2) were grown at 37 °C for 48–72 h under microaerophilic conditions on Columbia agar plates (Oxoid, UK) containing 5% horse blood (Nalgene, USA) and Dent's antibiotic supplement (Oxoid, UK). After growth, the biomass was collected with 50% phosphate-buffered saline (PBS; GIBCO®, Life-Technologies, UK) and the cells were harvested by centrifugation (18 000 × *g*, 1 min, 4 °C) and resuspended in buffer (0.02% Triton X100, 1% Tween 20 in 1× PBS, pH 7.4). Each 1 g (wet weight) of pelleted cells was resuspended in 10 mL of buffer and lysed by sonication (Sonics Vibra Cell™, USA) for 10 cycles of 1 min each. The insoluble material was separated by centrifugation (18 000 × *g*, 20 min, 4 °C) and the supernatant stored at −80 °C. Protein extracts were quantified by Bradford colorimetric assay (BioRad, USA).

Cloning the nap gene from H. pylori 26695

Cell pellet from *H. pylori* reference strain 26695 (ATCC 700392, USA) was obtained as described in the previous section. Genomic DNA was extracted, using a genomic DNA extraction kit (NZYTech, Portugal), according to the manufacturer's protocol, and quantified using a Quant-iT™ dsDNA Broad-Range kit (Molecular Probes™, Invitrogen, UK) using a Qubit™ Fluoro2meter (Invitrogen, UK) according to the manufacturer's protocol.

The *nap* gene sequence of *H. pylori* strain 26695 was obtained from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Primers used to amplify the *nap* gene included: in the forward primer the 5′ end information for the hydrophilic FLAG peptide tag (DYKDDDDK) and the restriction site for the PstI enzyme; in the reverse primer a restriction site for the Nhe I enzyme (Table 3). PCR was conducted with NZYTaq 2× Green

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Table 1. Countries (14 European, 15 Asian, 15 American, 4 Australian and 3 African) from where the 51 *H. pylori* strains were isolated and the corresponding 51 *nap* genes were sequenced

Continent	Country	Strain	Continent	Country	Strain
Europe	Spain	HUP-B14	Africa	South Africa	RHP901a
	France	B38			SouthAfrica7
	Italy	G27		Gambia	Gambia94/24
		G21	South and North America	Peru	Puno 135
	The Netherlands	5D			Shi169
		5A			Shi417
		2B			Shi470
		2A			Shi112
	Germany	P12			Cuz20
		P79			Sat464
	Sweden	HPAG1			SJM180
	UK	26 695			PeCan4
	Lithuania	Lithuania75			PeCan18
	Russia	A45		USA	J99
Asia	China	5060			EL37
		1811			8826
		XZ274		Canada	Aklavik86
	Japan	YS39			Aklavik117
		YS29	Oceania	Australia	SS1
		F16			NCTC 11637
		F30			NCTC 11639
		F32			
		F57			
		OK113			
		OK310			
	South Korea	51			
		52			
	India	Santal49			
		India7			

Table 2. *H. pylori* strains used in the NAP analysis by western blotting

Strain	Gastric disease	Geographical origin	
655/99	NUD	Portugal	Europe
173/00	NUD	Portugal	
1198/04	NUD	Portugal	
499/02	PUD	Portugal	
A3/90	GC	Portugal	
B23/99	GC	Portugal	
1152/04	NUD	Portugal	
26695	NUD	England	
EN32	PUD	England	
583	PUD	Sweden	
Mex 288	NUD	Mexico	North and South America
Arg 54	NUD	Argentina	
J99	PUD	USA	
SS1	PUD	Australia	Oceania
MONA	PUD	Egypt	Africa

Indicated are the country and the gastric disease of the person from which the bacteria were isolated (ulcer dyspepsia, NUD; gastric ulcer, PUD; gastric cancer, GC).

Table 3. Primer sequences used in *nap* gene amplification by PCR

Primer sequence (5′ → 3′)
Forward: CTAGCTAGCGACTACAAAGACGATGACGACAAGTAGATGAAAACATTTGAAATTC
Reverse: AACTGCAGTTAAGCCAAATGGGCTTGC

Master Mix (NZYTech, Portugal), according to a denaturation cycle (5 min at 95 °C), 30 cycles (including a denaturation for 30 s at 95 °C, an annealing for 30 s at 58 °C and an extension for 40 s at 72 °C) and a final extension cycle for 10 min at 72 °C. A final 479 bp fragment was obtained after PCR amplification.

The 479 bp amplification product was purified using a DNA NZYGelpure kit (NZYTech, Portugal), according to the manufacturer's protocol, and was subsequently cloned into the expression vector pQE30 (Qiagen GmbH, Germany) and subsequently used to transform *Escherichia coli* NZY5*α* (NZYTech, Portugal). All the constructs were confirmed by DNA sequencing.

Expression and purification of recombinant NAP

Recombinant *E. coli* was grown in an orbital incubator at 37 °C in LB medium with 100 μg mL^{-1} of ampicillin until an optical

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absorbance of 0.6 at 600 nm. Subsequently, recombinant NAP expression was induced by 1 mmol L^{-1} IPTG addition and growth continued for a further 3 h. Bacteria were harvested by centrifugation (10 000 × *g*, 15 min, 4 °C), and resuspended in binding buffer under denaturing conditions (8 mol L^{-1} urea, 500 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris–HCl, 1 mmol L^{-1} *β*-mercaptoethanol, 5 mmol L^{-1} imidazole, pH 8.0). The cells were lysed by sonication (Sonics Vibra Cell™, USA) for 10 cycles of 1 min each and the final supernatant collected after centrifugation (12 000 × *g*, 4 °C, 1 h). The supernatant was filtered using a 0.45 μm nonsterile filter, followed by NAP purification by affinity chromatography conducted under denaturing conditions on a HisGraviTrap HP column (GE Healthcare, UK) charged with nickel according to the manufacturer's instructions. Purified NAP was lyophilized before storing. NAP expression and purification were confirmed by Coomassie staining electrophoresis gels and western blotting analysis as previously described by Azevedo *et al.*[32]

Generation of NAP anti-sera

Female BALB/c mice ($n = 2$ per group), 6–8 weeks old, provided with food and drink *ad libitum*, were used in the *in vivo* studies, which were performed in strict accordance with Directive of 24 November (no. 86/609 EEC), the Portuguese laws DR no. 31/92, DR 153 I-A 67/92, and all following legislations. Doses of 50 μg of purified recombinant NAP in sterile 10 mmol L^{-1} PBS (pH 7.4) were added intramuscularly to mice on day 1 and boosts on days 17 and 45. Blood samples were collected from the tail vein 67 days after the first NAP injection and sera was separated by centrifugation (18 000 × *g*, 5 min, 4 °C; Allegra 64R, Beckman, USA) and stored at −20 °C.

Western blotting to detect NAP from diverse *H. pylori* strains

The protein extracts of 15 strains of *H. pylori* were separated by 12% SDS-PAGE, transferred onto poly(vinylidene difluoride) membranes (GE Healthcare, UK) and blotted with mice serum with polyclonal NAP antibodies (anti-NAP) obtained as described in the previous section. Goat-anti-mouse IgG antibody coupled to alkaline phosphatase (Sigma Aldrich, Germany) was used as secondary antibody. Membranes were probed in accordance with the SIGMA FAST™ BCIP and NBT substrate protocol (Sigma Aldrich, Germany). Protein concentration was determined using the Bradford colorimetric assay (BioRad, USA).

RESULTS

NAP conservation

From the alignment of the 51 NAP sequences, a high conservation was observed (83%), where the first 45 amino acid residues were 100% conserved (Fig. 1). Among the 144 amino acid residues, only 17 present polymorphisms (Table 4). This is according to previous observations reported by Calado,[23] where the high *nap* gene variability (where 88% of the 51 sequences represented a new allele) was associated with a high proportion of synonymous substitutions (Ks) in relation to non-synonymous substitutions (Ka), with Ks/Ka = 11.

NAP antigenicity

The antigenicity profile of the consensus NAP was predicted based on diverse algorithms (Fig. 2): the Jameson–Wolf method, predicting antigenic determinants by considering hydrophilicity, surface probability and flexibility of the protein backbone; the

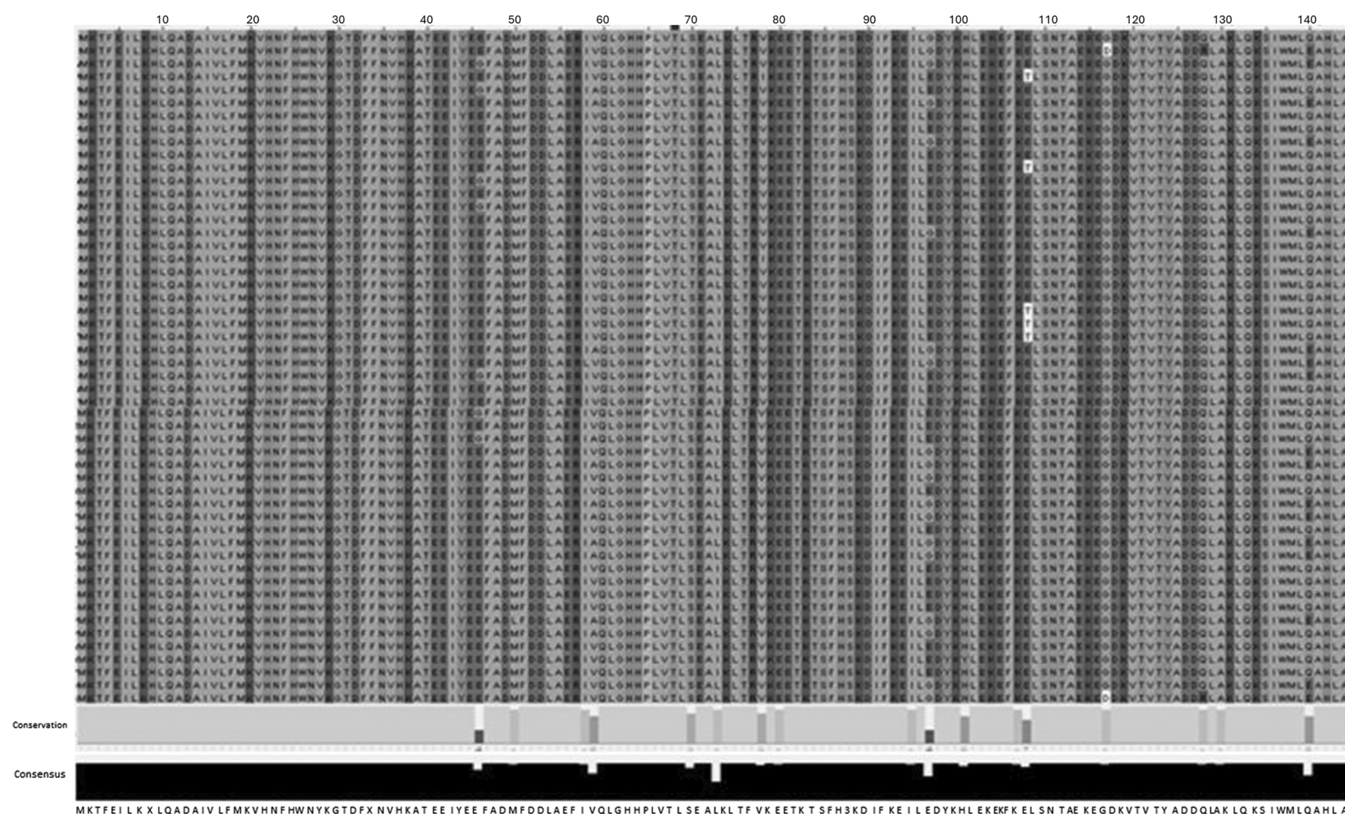

Figure 1. Alignment of 51 NAP sequences, highlighting the conservation along the NAP sequences and the consensus sequence.

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Table 4. The 17 positions of amino acid residues (AR) of the consensus NAP presenting polymorphisms among 51 sequences

AR position on consensus sequence	AR of consensus sequence (side chain property)	Number of sequences that presented a different AR at that position, and the corresponding AR usually encountered after the most common
46	Glutamic acid (negative)	7 Glycine (non-polar)
50	Methionine (non-polar)	1 Leucine (non-polar)
58	Isoleucine (non-polar)	1 Leucine (non-polar)
59	Valine (non-polar)	13 Alanine (non-polar)
70	Serine (uncharged polar)	6 Threonine (uncharged polar)
73	Leucine (non-polar)	24 Isoleucine (non-polar)
78	Valine (non-polar)	2 Isoleucine (non-polar)
80	Glutamic acid (negative)	1 Aspartic acid (negative)
95	Isoleucine (non-polar)	1 Phenylalanine (non-polar)
97	Glutamic acid (negative)	19 Glycine (non-polar)
101	Histidine (positive)	2 Tyrosine (uncharged polar)
107	Lysine (positive)	1 Glutamic acid (negative)
108	Glutamic acid (negative)	7 Threonine (uncharged polar)
117	Glycine (non-polar)	2 Aspartic acid (negative)
128	Glutamine (uncharged polar)	2 Arginine (positive)
130	Alanine (non-polar)	1 Valine (non-polar)
140	Glutamine (uncharged polar)	17 Glutamic acid (negative)

At each of 17 positions are indicated the AR of the consensus protein, the number of sequences that present a different AR and the corresponding most common AR after the one present at the consensus sequence.

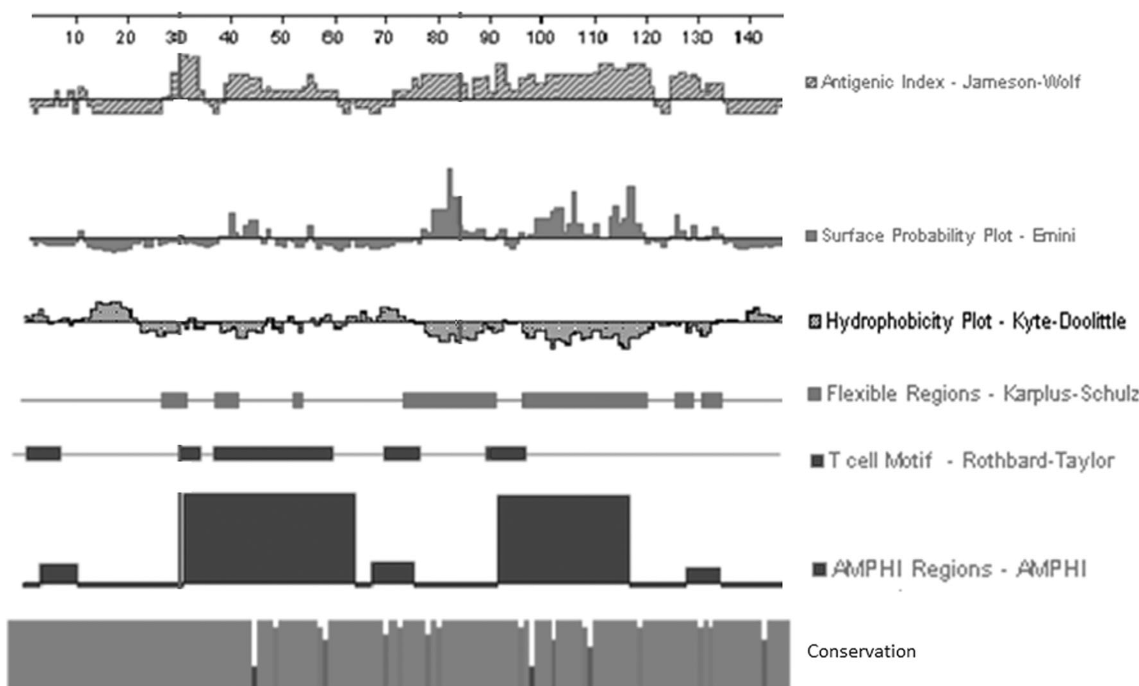

Figure 2. Prediction of NAP antigenicity profile based on the Jameson–Wolf method, Rothbard–Taylor method and AMPHI method for the consensus sequence and its conservation among 51 sequences.

Rothbard–Taylor method that predicts 80% of T antigenic determinants, by comparison with known common sequence motifs; and the AMPHI method, predicting Th based on the identification of amphipathic helices.

As expected, the regions of the NAP less conserved were theoretically more antigenic (Fig. 2), since regions that usually are more prone to be recognized by the host immune system, i.e. more antigenic, are usually less conserved as a way for the bacteria to evade the host immune system.

A total of 17 polymorphic regions were identified over the 51 sequences. From this, only the following 5 from the 51 NAPs (i.e. 10%) presented a slightly different antigenic profile in relation

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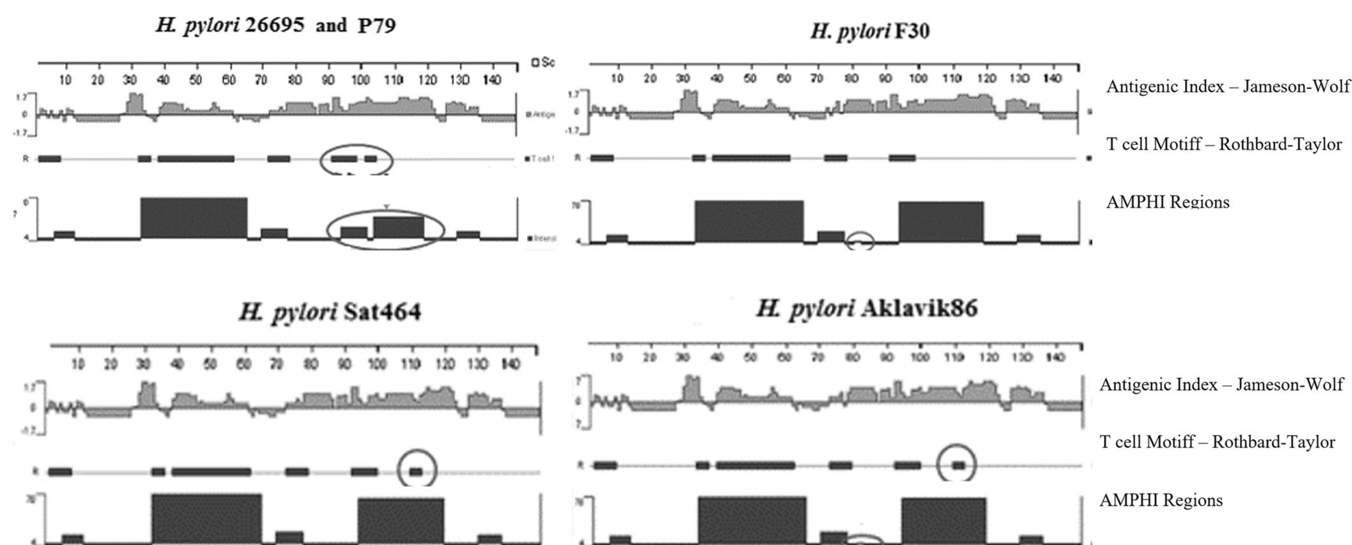

Figure 3. Antigenic regions of NAP from strains 26695 and P79, F30, Sat464 and Aklavik86. The regions that pointed a new antigenic profile in relation to the consensus sequence are highlighted by a circle.

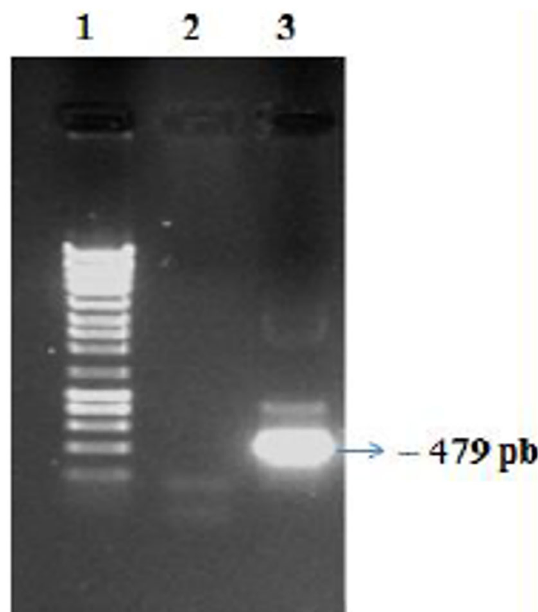

Figure 4. Agarose gel electrophoresis of the fragment amplified by PCR of the *nap* gene from the pQE30-nap plasmid: (1) molecular weight marker NZYDNA Ladder III; (2) PCR product obtained using the non-recombinant pQE30 plasmid; (3) PCR product obtained using the recombinant pQE30-nap plasmid, i.e. containing the *nap* gene.

to the consensus sequence (Fig. 3): 2 strains from Europe, 1 from Japan, 1 from Canada and 1 from Peru. In these 5 NAPs, the antigenic profile changed due to amino acid residue alterations at positions 78, 101 and 108 (Fig. 3). The 5 NAPs with slightly different antigenic profiles were as follows. NAP from F30 strain isolated from a Japanese patient presenting two polymorphisms in relation to the consensus sequence: Thr70 that did not modify the antigenicity and Ile78 that led to a new AMPHI motif. The strains from Canada (Aklavik86) and Peru (Sat464) presented between two and three polymorphic sites. Both presented Ile73 that did not result in a different antigenic profile, while Thr108 resulted in a new T-cell motif predicted by the Rothbard–Taylor method. The Canadian strain also presented the polymorphism Ile78 that led to a new Th motif predicted by the AMPHI method. The two European strains, 26695 and P79, isolated from a UK patient and a German patient, respectively, presented NAPs with the same polymorphism: Ile73 that did not result in a different antigenic profile and Tyr101 that predicts a new Th motif by the AMPHI method.

Development of recombinant NAP and generation of corresponding polyclonal antibodies

Before evaluating if NAP from diverse strains is recognized by the same NAP polyclonal antibody, it was necessary to generate serum with polyclonal antibodies. For that, a recombinant NAP expression system was developed, which was subsequently used to produce anti-serum in mice.

The *nap* gene from *H. pylori* 26695 strain was cloned and produced in an *E. coli* expression system based on a pQE30 plasmid containing a His-tag. The *nap* correct cloning in pQE30 was confirmed by gene sequence and the dimension of the fragment in the electrophoresis obtained after PCR of the plasmid (Fig. 4). Extracts obtained from recombinant *E. coli* (i.e. containing pQE30-nap), along diverse induction periods of the *nap* gene expression, were analyzed by western blotting using an anti-His monoclonal antibody (MAB) (Fig. 5(A)). NAP was detected in all *E. coli* samples even at the beginning of the *nap* expression induction period. The *nap* basal expression at the beginning of induction can result from the gene promoter activation by other routes.[33] The maximum NAP production was observed after 3 h of induction in accordance with other authors.[34]

The predicted dimension of recombinant NAP, including the 1.01 kDa His-tag, is ∼19 kDa. However, observed in all *E. coli* samples were NAP fragments of ∼14 kDa, and NAP multimers of ∼40 kDa and ∼216 kDa (Fig. 5(A)). NAP from *E. coli* protein extracts was subsequently purified by IMAC, being observed again in western blotting using anti-His MAB a major band equivalent to a NAP dimer, i.e. of ∼42 kDa, and a band near the 19 kDa marker, probably due to the NAP monomer (Fig. 5(B)). The small-dimension NAP fragments observed before and after NAP

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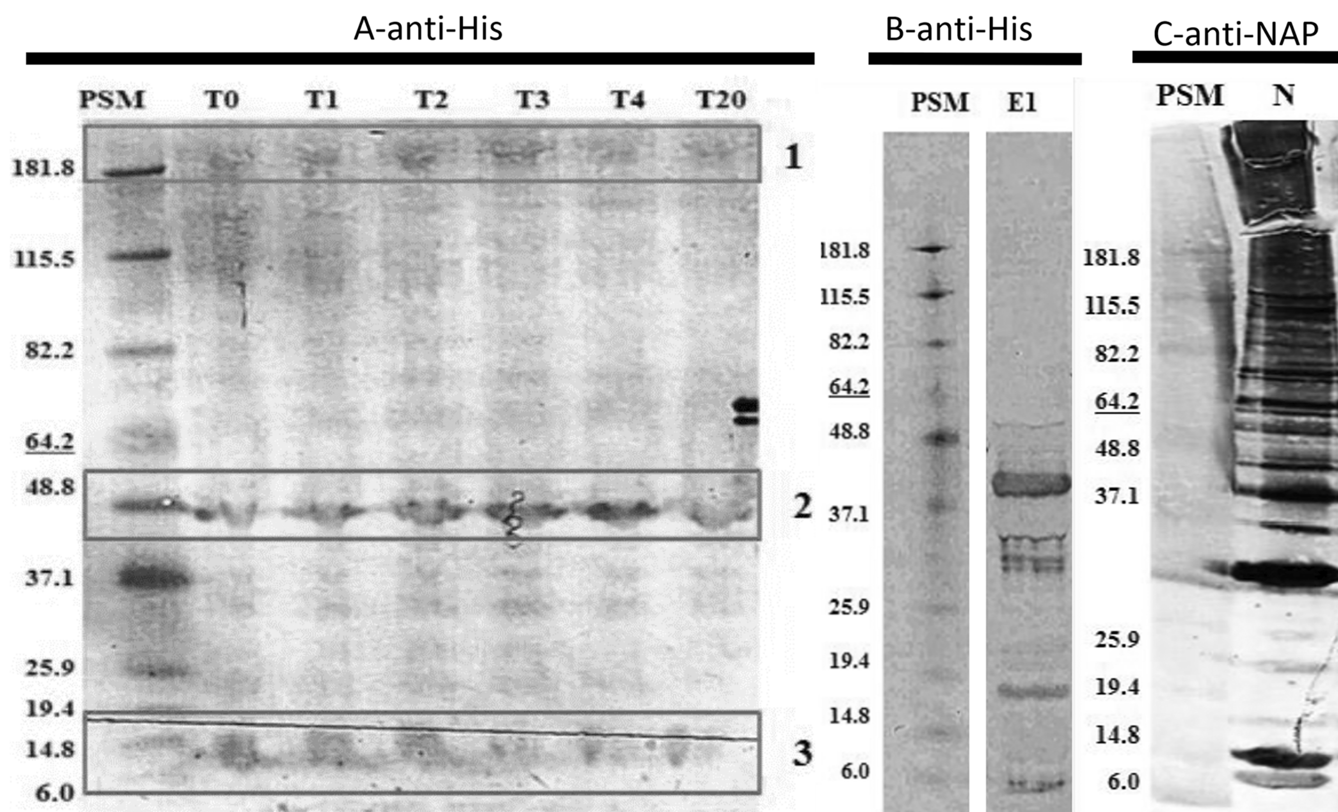

Figure 5. Western blotting. (A, B) Conducted with anti-His MAB. PSM, molecular weight marker. (A) Samples from recombinant *E. coli* (with pQE30-nap) extracts obtained after 0, 1, 3, 3, 4 and 20 h of *nap* expression induction. (B) Elution fraction of IMAC with *E. coli* protein extract obtained after 3 h of *nap* expression induction. (C) Conducted with mouse serum immunized with recombinant NAP, against the recombinant NAP.

purification can result from NAP hydrolysis or NAP partial expression. A small-dimension NAP of 12 kDa was, for example, also observed by McAtee *et al*.[35]

The serum obtained from immunized mice with the recombinant NAP was tested against the recombinant NAP itself (Fig. 5(C)). Diverse bands were recognized, including bands with high dimension molecular weight, a band at ~43 kDa, as previously observed, with the most intense band at ~29 kDa. This band was present in western blotting conducted with anti-His (Fig. 5(A),(B)) but was less intense compared to the ~40 kDa fragment. The ~29 kDa fragment probably resulted from the association between NAP fragments that occurred during NAP storage after its purification, which included a lyophilization step.

Western blotting to detect NAP from diverse *H. pylori* strains

It was evaluated if diverse NAP sequences, i.e. NAPs obtained from different strains, would be recognized by antibodies developed against a specific NAP. For that, western blotting was conducted using serum from immunized mice with NAP from *H. pylori* 26695. Protein extracts from 15 *H. pylori* strains were evaluated, isolated from the following patients: 7 Portuguese presenting diverse gastric diseases, 2 English, 1 Swedish, 1 Egyptian, 1 Australian, 1 American (USA), 1 Mexican and 1 Argentine (Table 2). NAP was detected in diverse protein extracts at a band at ~30 kDa (Fig. 6), as previously observed in Fig. 5(C).

From the 15 strains evaluated, only one protein extract (from 1152/04 strain) was not recognized by the serum (Fig. 6). The lack of recognition of protein extracts from one of the 15 strains most probably reflects the inexistence of the *nap* gene since few local alterations were observed on the antigenic profile among the 51 sequences.

Ten of the protein extracts were recognized strongly (from B23/95, 26695, J99, 499/02, 1198/04, A3/90, EN32, 655/99 and Mex288 strains), in relation to four other protein extracts (from MONA, 583, SS1 and Arg strains) (Fig. 6). Since the same protein quantity was applied in the corresponding SDS-PAGE, this results most probably from the different *nap* expression levels among the 14 strains.

DISCUSSION

NAP conservation

The high NAP conservation (83%), and the high proportion of synonymous substitutions (Ks/Ka = 11), points to an equilibrium between the need for antigenic diversity, as a mechanism for bacteria to escape the host immune system, and the maintenance of the NAP function. The much lower Ks/Ka observed with other virulence factors such as *cagA* and *vacA* (with Ks/Ka values between 2.2 and 6.9 among worldwide bacteria)[23] points to NAP as a good target for diagnostic and vaccine design in relation to other immunogenic virulence factors.

From the 17 NAP polymorphic sites identified, 9 were also present in other studies,[36,37] and the following were, from our knowledge, identified for the first time in the present work: 50, 59, 78, 95, 107, 108, 117, 128 and 130. Only the polymorphism at position 8 identified by Grandi[37] was not detected among the present 51 sequences. Grandi[37] reported two NAP sequences that differed

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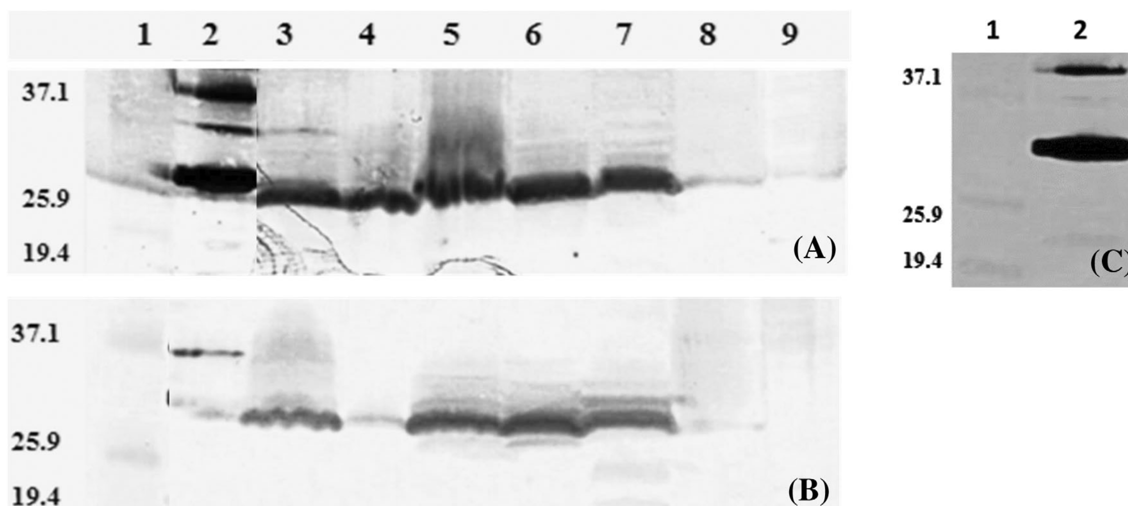

Figure 6. Western blotting performed with anti-NAP mouse serum, immunized with NAP from *H. pylori* 26695, and applied to NAP recognized from protein extracts from diverse *H. pylori* strains. (A) Lane 1, molecular weight markers; lane 2, purified recombinant NAP; lanes 3–9, extracts from *H. pylori* strains B23/95, 26695, J99, 499/02, 1198/04, MONA and 583, respectively. (B) Lane 1, molecular weight markers; lanes 2–9, extracts from *H. pylori* strains 173/00, A3/90, SS1, EN32, 655/99, Mex288, Arg and 1152/04, respectively. (C) Lane 1, molecular weight markers; lane 2, purified NAP.

on amino acid residues 8, 58 and 80, and on two other NAP sequences in positions 8, 73, 97, 101 and 140. Polymorphisms at positions 46, 70 and 73 were also previously observed by Yokota *et al.*,[36] who evaluated 49 NAP sequences from strains isolated from Japanese patients. The percentage of each polymorphism was different from those herein detected, most probably due to a more homogeneous *H. pylori* population. In the present study only 8 out of 51 strains were Japanese. As examples, the following differences were observed: 84% and 86% presented a glutamic acid instead of a glycine at position 46, 51% and 88% presented a serine instead of a threonine at position 70, and 76% and 52% presented a leucine instead of an isoleucine at position 73 in the Yokota *et al.*[36] study and in the present study, respectively.

NAP antigenicity

As expected, the regions of the NAP less conserved were theoretically more antigenic. For example, regions between amino acid residues 69 and 114, usually associated with NAP activity,[38] are antigenic and less conserved. The antigenic profile of the consensus sequence is according to the region between 70 and 90, that presented a very low AMPHI value, eliciting a very poor immune response in BALB/c mice.[39] The amino acid residue at position 101, included in a region predicting AMPH11 and T-cell motifs, is according to the fact that it is critical for NAP recognition by MAB-23C8.[39] Residues at positions 25, 37, 52 and 134 that are critical for NAP dodecamer formation[38] are conserved, most probably because their variations would critically impair the protein function.

Despite the 17 polymorphic sites, only 5 NAPs (i.e. 10%) presented a different antigenicity profile. The polymorphism at position 101 is in line with Iankov *et al.*[39] observations, where this residue was critical for NAP recognition by MAB-23C8. NAP presenting His101 was not recognized by this MAB. Since only 10% of the NAP presented slightly different antigenic profiles, most probably polyclonal antibodies raised against a specific NAP sequence will recognize NAP from other strains. Furthermore, the NAP regions selected to generate MABs should be carefully selected, to avoid polymorphic regions with impact on the antigenicity profile.

Development of recombinant NAP and generation of corresponding polyclonal antibodies

The NAP multimers observed in western blotting can result partially from the characteristics of the expression system, since *E. coli* tends to produce recombinant proteins in insoluble aggregates in inclusion bodies, resulting in the need for high concentration of chaotropes like urea and guanidine hydrochloride, which leads to protein denaturation, but can often lead to polypeptide aggregation during the refolding process.[40] Furthermore, NAP naturally tends to form multimers, native NAP being present as a dodecamer.[2] The NAP dimer was also observed by Tonello *et al.*[3] in SDS-PAGE using similar production conditions.

Western blotting to detect NAP from diverse *H. pylori* strains

The high percentage of bacterial strains recognized by the NAP polyclonal antibodies (93%) is according to 90% of NAP presenting the same antigenic profile, and in accord with other authors. For example, Tang *et al.*[18] observed that 135 out of 141 patients (i.e. 95.7%) presented IgG that recognized in an ELISA a specific NAP. In two-dimensional immunoproteomic studies, usually NAP recognition is lower most probably resulting from a high dilution of the serum, which are generally used to recognize *H. pylori* proteins produced in greater amounts than NAP, such as Ure, CagA and VacA. For example, the NAP of a specific strain was recognized by 10 out of 16 human sera (62.5%)[41]; the NAP of 18 out of 23 protein extracts (78%) was recognized by a specific serum.[42] Five different human sera from infected patients were tested against protein extracts from 3 different *H. pylori* strains, where only 5 out of the total of 15 combinations (i.e. 33.5%) between protein extracts and serum were detected.[43]

CONCLUSION

The current results from the theoretically predicted antigenic profile (based on 51 sequences retrieved worldwide), and from

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western blotting (based on extracts from 15 strains also isolated from strains of different continents) point to the following:

- Polyclonal antibodies generated against a specific NAP most probably will recognize NAPs from other strains.
- The whole NAP or a large NAP fragment considered for vaccine construction most probably will generate an immune response that will recognize NAP from the *H. pylori* population.
- Since local polymorphisms of the NAP sequence can also lead to local antigenic variations, diagnostic tests based on NAP MABs could result in non-recognition of other NAPs, leading to false negatives.
- Vaccine constructions based on small NAP epitopes can result in an immune response not recognizing a specific NAP.
- For designing MABs for diagnostics or selecting epitopes for vaccine design, it is critical to select a NAP region maximizing conservation and antigenicity, such as, for example, the region between residues 30 and 85.

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All authors contributed to the experimental realization, discussion and manuscript writing.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interests.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All experiments with mice were performed in strict accordance with Directive of 24 November (no. 86/609 EEC) and the Portuguese laws DR no. 31/92, DR 153 I-A 67/92.

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