Analysis of *iceA* genotypes in South African *Helicobacter pylori* strains and relationship to clinically significant disease

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Abstract

Background—South African *Helicobacter pylori* isolates are characterised by the universal presence of *cagA* but have differences in vacuolating cytotoxin gene (*vacA*) alleles which correlate with clinically significant disease. However, the candidate virulence marker gene *iceA* has not been investigated.

Aim—To characterise the genetic organisation and heterogeneity of *iceA* genotypes in different South African clinical isolates.

Patients and methods—We studied *H pylori* strains isolated from 86 dyspeptic patients (30 with peptic ulcer disease (PUD), 19 with distal gastric adenocarcinoma (GC), and 37 with non-erosive gastritis) for the presence of *iceA1* or *iceA2* genes, and for differences in the genetic organisation of *iceA2* by polymerase chain reaction, Southern hybridisation analysis, and sequencing.

Results—Genetic analysis of *iceA1* demonstrated significant homology (92–95%) with the USA type strain 26695 and probably functions as a transcriptional regulator, while a novel variant (*iceA2D'*) of *iceA2* and marked differences in predicted protein secondary structure of the *iceA2* protein were defined. *iceA1* was detected in 68% and *iceA2* in 80% of all clinical isolates. Although approximately 40% of patients had both strains, a higher prevalence ($p < 0.01$) of GC patients were infected with *iceA1* isolates which were invariably *vacA* s1/*iceA1* ($p < 0.005$ v gastritis). Isolates from PUD patients were distinguished by the structurally altered *iceA2D* variant (53%; $p < 0.03$ v gastritis) while the *iceA2C* variant distinguished isolates from patients with gastritis alone (67%; $p < 0.005$ v PUD).

Conclusion—In this study, an association between *iceA1* and GC was noted while differences in variants of *iceA2* differentiated between PUD and gastritis alone. Combination analyses of *iceA* genotypes and *vacA* alleles supported these associations.

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Keywords: adenocarcinoma; gastritis; *Helicobacter pylori*; *iceA*; peptic ulceration; protein prediction; sequencing; South Africa

Helicobacter pylori induces gastric inflammation in virtually all colonised individuals and such

gastritis increases the risk of peptic ulcer disease (PUD) and non-cardia/distal gastric adenocarcinoma (GC).^{1–3} However, only a minority of patients carrying *H pylori* develop clinical sequelae, suggesting that particular bacterial products may contribute to pathogenesis.⁴ Recently, a novel *H pylori* gene *iceA* was identified following transcriptional upregulation on contact with gastric epithelial cells.⁵ *iceA* exists as two distinct genotypes, *iceA1* and *iceA2*, and only *iceA1* RNA is induced following adherence in vitro.⁵ *H pylori iceA1* demonstrates strong homology to a restriction endonuclease *nlaiIIR* in *Neisseria lactamica*,⁶ and in vivo carriage of *H pylori iceA1* strains has been reported to be associated with peptic ulceration and enhanced acute neutrophilic infiltration.^{5,7,8} However, linkage between the *iceA1* genotype and ulcer disease is not universal,⁹ and thus may be population dependent.

In contrast with *iceA1*, *iceA2* has no significant homology to known proteins and its structure reveals patterns of repeated protein cassettes. Recently, the genetic organisation and sequence heterogeneity of *iceA2* has been studied,⁶ revealing five distinct *iceA2* subtypes. While *iceA2* strains are more prevalent among patients with asymptomatic gastritis and non-ulcer dyspepsia,⁷ a statistically significant relationship between *iceA2* subtypes and disease has not yet been defined.

South African *H pylori* isolates are characterised by the universal presence of *cagA* but differences in the 3' region of *cagA* and *vacA* alleles correlate significantly with clinical disease.^{10,11} Our hypothesis was that *H pylori* isolates from South African patients may also exhibit variability in their *iceA* alleles and that this variability may be related both to clinical outcome and to *vacA* allelic status.

Materials and methods

H PYLORI STRAINS AND DNA

H pylori isolates, cultured from single biopsies from 86 clinically defined patients (37 patients with gastritis alone, 30 patients with PUD, and 19 patients with GC), were examined. Fifty six of the resultant isolates had *vacA* and *cagA* determined in a previous study.¹¹ Putative clonal isolates were obtained from eight of the parent strains by serially reculturing single colonies twice. Reference strains 26695 (*iceA1*)

Abbreviations used in this paper: PUD, peptic ulcer disease; GC, gastric adenocarcinoma; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; DAS, dense alignment surface; SSC, sodium chloride/sodium citrate.

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Figure 1 Primers, sites, and amplicon sizes for *iceA1* (HP1209, GenBank Accession No AE000511) and *iceA2* (JHP1132, GenBank Accession No AE001541) (see text for details).

isolated from a patient in the UK with gastritis¹² and J99 (*iceA2*) isolated from a USA patient with duodenal ulcer disease¹³ were used as *iceA1* and *iceA2* positive and negative controls, respectively, in this study.

AMPLIFICATION OF *iceA* BY PCR

Polymerase chain reactions (PCRs) were performed as described previously^{5, 11} but a final [MgCl₂] concentration of 3.5 mM was used for the *iceA1* PCRs. For amplification of the *iceA1* allele, forward primer *IceA1F* (5'-CGTTGG GTAAGCGTTACAGAATTT) and reverse primer *IceA1R* (5'-TCATTGTATATCCTAT CATTACAAG) yielded a fragment of 558 bp (fig 1). For *iceA2*, primers *IceA2F* (5'-GTTG TCGTTGTTTAAATGAA) and *IceA2R* (5'-GTCTTAAACCCACGATTAAA) yielded a fragment of 120 bp. PCR was performed in a Sprint PCR (Hybaid, South Africa) under the following conditions: four minutes of preincubation at 94°C followed by 30 cycles of one minute at 94°C, one minute at 62°C (*iceA1*) or 53°C (*iceA2*), and one minute at 72°C. Final extension was performed for 10 minutes at 72°C.

Additional genotype specific PCR assays that used primers for *iceA1* (*IceA1F5* (5'-GTG TTTTAAACCAAAGTATC) and *IceA1R4* (5'-CTATAGCCASTYTCTTTGCA)) and primers flanking the *iceA2* internal cassette were performed to confirm the presence of the genes and to identify the size and subtype of the *iceA2* allele.⁷ Primers *IceA2F6* (5'-GTTG GGTATATCACAATTTAT) and *IceA2R5* (5'-TTRCCCTATTTTCTAGTAGGT) yielded a fragment of 229, 334, or 439 bp according to the existence of repeated sequences of 105 nucleotides. PCR was performed under the following conditions: four minutes of preincubation at 94°C followed by 30 cycles of one minute at 94°C, one minute at 53°C (*iceA1* and *iceA2*), and one minute at 72°C. Final extension was performed for 10 minutes at 72°C. *iceA* amplicons were examined by electrophoresis on 1% agarose gels according to standard procedures.¹¹

SEQUENCING OF *iceA1* AND *iceA2*

The complete *iceA1* gene, from the upstream *cysE* (HP1210) to the downstream *hpyIM* (HP1208), was amplified using F1 (5'-GGG TGCGATTTGCGTGGGCGATG) and R10 (5'-GATCATGGCCTACAACCGCATGGA),

as described previously,¹⁴ while *iceA2* was amplified using primer sets *IceA2F6/R5*. PCR products were gel extracted (QIAEX II gel extraction kit; Qiagen, Cape Town, South Africa) and sequenced on an ABI PRISM 377 automated sequencer (ABI, Foster City, California, USA) using the ABI PRISM BigDye terminator cycle sequencing reagent kit with AmpliTaq DNA polymerase FS (PE Biosystems, Johannesburg, South Africa), as described previously.¹⁵ PCR and direct sequencing were performed at least twice to determine DNA sequences for each strain.

SOUTHERN HYBRIDISATION ANALYSIS

Southern hybridisation analysis was performed essentially as described previously.¹⁶ Chromosomal DNA digested with *Bgl*II was subjected to electrophoresis on a 0.8% Tris acetate/EDTA agarose gel, transferred to a Hybond-N⁺ membrane (AP Biotech, Piscataway, New Jersey, USA) by capillary transfer, and UV cross linked. Prehybridisation was performed using 6× sodium chloride/sodium citrate (SSC), 5× Denhardt's, 0.5% sodium dodecyl sulphate (SDS), and 100 µg/ml of sheared salmon sperm DNA at 60°C for three hours. Buffer was replaced with fresh buffer containing denatured probe and hybridisation proceeded at 60°C for 18 hours. Membranes were washed for five minutes with 2× SSC, 0.5% SDS at room temperature, 15 minutes with 2× SSC, 0.1% SDS at room temperature, two hours with 0.2% SSC, 0.5% SDS at 60°C, and 30 minutes with 0.2% SSC, 0.5% SDS at 60°C. The membranes were then subjected to autoradiography for detection.

Probes for *iceA1* and *iceA2* were generated by PCR using primer pairs *IceA1F5/IceA1R4* and *IceA2F6/IceA2R5*, respectively, as described previously,⁷ with 26695 and J99 chromosomal DNA as respective templates. Products were purified using QiaQuick PCR purification columns (Qiagen, Valencia, California, USA) and labelled with [α -³²P]dCTP using the PRIME-IT RmT labelling kit as recommended by the manufacturer (Stratagene, La Jolla, California, USA). Unincorporated nucleotides were removed using ProbeQuant G-50 micro-columns according to the manufacturer's instructions (AP Biotech). Probe was denatured by boiling for five minutes and then placed on ice prior to hybridisation.

COMPUTER ANALYSIS

DNA sequences were analysed using the National Center for Biotechnology Information (NCBI) server (USA) and consensus secondary structure prediction for proteins was obtained using the META Predict Protein (which includes the SignalP, Jpred, TopPred, and DAS Servers (Columbia University, USA)).¹⁷⁻²⁰ SignalP predicts the presence and location of signal peptide cleavage sites, Jpred predicts secondary structures, TopPred is useful for prediction of the location and orientation of transmembrane sequences through the use of hydrophobicity patterns and by applying the "positive inside" rule, while DAS predicts the location of transmembrane using hydrophobicity patterns. Internet based

Figure 2 Alignment of nucleotide sequences of 60190, 26695, and five South African strains showing the 3' end of *cysE*, the complete *iceA1* gene (ORF1 and ORF2), intervening sequences (1–3), and the 5' end of *hypIM* putative start codon. *ATG*₆₁₇ (italicised and boxed) is the start codon for *nlaiiIR* and is only conserved in 3/5 strains. None of the strains however is predicted to produce the *NLAIH* homologue of 228 amino acids. *ATG*₉₁₉ (boxed/bold), the start codon for *iceA1*, is conserved in all five South African isolates, as are the upstream transcription boxes (at –35 and –10). Predicted *iceA1* proteins range in size from 128 to 136 amino acids, except for strain 54 (frameshift results in a protein of 53 amino acids). All isolates shared substantial protein homology (93–96%) with HP1209. Sequence gaps are indicated by dashes (-).

searches were performed at NCBI, the Institute for Genome Research (TIGR, Maryland, USA), and Astra-Zeneca (Boston, USA).

STATISTICS

Data were examined using the χ^2 test or Fisher's exact test as appropriate. Probability levels of <0.05 were considered statistically significant.

Results

iceA1 SEQUENCE ANALYSIS

In a preliminary study, in order to establish the composition of *iceA* gene products in South African *H. pylori* isolates, *iceA1* (primers F1/R10: 952 bp) and *iceA2* (primers F6/R5: 229–334 bp) were sequenced.

Five isolates with the *iceA1* genotype (two from patients with gastritis alone, one from a patient with peptic ulceration, and two from patients with gastric cancer) were sequenced and compared with the type strains 60190 (GenBank Accession No U43917) and 26695. Alignment of these sequences revealed the presence of a putative conserved initiation codon at position *ATG*₉₁₉ in all five South African strains

as well as conserved upstream transcription initiation sites, as previously reported⁶ (fig 2). Predicted proteins ranged from the expected 128 amino acids to 136 amino acids in four strains. No full length *NLAIH* homologues were predicted. These results suggested that the *iceA1* gene does not encode a functional restriction endonuclease but probably acts as a transcriptional regulator.¹⁴

iceA2 SEQUENCE ANALYSIS

Seven isolates with the *iceA2* genotype (two from patients with gastritis alone, two from patients with PUD, and three from patients with GC) were sequenced and compared with the predicted gene (JHP1132) present at the same locus in strain J99 (fig 3).

Prior to our analysis, five different variants (A–E) based on size (24, 59, 94, or 129 amino acids) and/or sequence of *iceA2* had been described.⁶ The *iceA2* gene (JHP1132) from strain J99 which conforms to the structure of repeats (14–13–16_{2B}–6–10) demonstrated by Figueiredo and colleagues⁶ generates a predicted protein of 59 amino acids and is an example of the *iceA2B* subtype.⁶ None of the South African isolates sequenced in this series had either a 2A structure (14–13–16–6–10) or a

Figure 3 Amino acid alignment of *iceA2* genotypes from seven South African isolates and homology with JHP1132. The different peptide domains are boxed and the amino acid number indicated. Asterisks (*) represent gaps in sequence homology.

2B structure. Two isolates (Hp27 and 215) however had a 14-13-16_{2C}-6-10 motif and were therefore classified as *iceA2C* subtypes. Four isolates had the pattern 14-13-16_{2B}-6-13-16_{2C}-6-10 and are examples of the *iceA2D* subtype. These subtypes would be predicted to generate proteins of 94 amino acids but one isolate (Ca102) had an inframe three nucleotide deletion within cassette 16_{2B} which resulted in a putative protein truncation of 93 amino acids. Sequence analysis of a fifth isolate (1715) demonstrated a novel variant of the *iceA2D* subtype that was typified by the sequence 14-13-16_{2C}-6-13-16_{2C}-6-10 (sequence identity (71%) and shared residues (80%) with *iceA2D*).

Analysis of the protein signal sequence, secondary structures, and topology for the South African *iceA2* variants is provided in table 1. Of note are the observations that these proteins do not have a typical prokaryote signal sequence

(from the SignalP server), and the N terminus appears to be embedded within the cell membrane. The TopPred topology program demonstrated that variants 2B and 2C had no predicted transmembrane regions while 2D and the novel 2D variant (2D') had a single putative transmembrane region with scores of 0.758 and 0.615, respectively (fig 4).¹⁹ The dense alignment surface (DAS) method confirmed that variant 2B had no transmembrane region but suggested a possible transmembrane region in 2C and confirmed two putative transmembrane regions in 2D and the 2D' variant.²⁰ Variant 2B can be classified as a mixed class protein but 2C, 2D, and 2D' are all *all beta* proteins.

CLINICAL RELEVANCE OF *iceA* GENOTYPES

After validating the PCR protocol and examining the structure of *iceA1* and *iceA2*, PCR was performed in clinical isolates and products were analysed in terms of the distribution of genotypes. Fifty four isolates were positive for either *iceA1* or *iceA2* using previously described primers *IceA1F/1R* and *IceA2F/2R*,⁵ 54 isolates were positive for both *iceA1* and *iceA2*, and one isolate did not yield any PCR product for either of the *iceA* genotypes. Amplicons of the respective sizes were obtained from reference strains 26695 and J99 as expected. Twenty one of the 54 *iceA1*⁺/*iceA2*⁺ isolates were re-examined with different *iceA* primer sets (F5/R4 (*iceA1*) and F6/R5 (*iceA2*))^{6,7} to determine whether this "mixed genotype" was a consistent finding. All 21 (100%) were positive for both genotypes, indicating that these primer sets are concordant. These isolates each had a single *vacA* allele, a single *cagA* 3' amplicon, which suggested the presence of multiple *iceA* genotypes in these strains.

We then examined the presence of these putative "mixed genotypes" further by performing repeat PCR (using two primer sets) and Southern hybridisation in multiply recultured single colonies from eight *iceA1*⁺/*iceA2*⁺ parent strains. Seven of the eight isolates had a single *iceA* allele by both PCR and Southern analysis (fig 5). No hybridisation was seen in one isolate. A single PCR band using primer sets *IceA2F6/IceA2R5* (which identifies *iceA2* sizes and subtypes) and a single Southern band was seen in the three clonal strains that were *iceA2*⁺. These data signify the presence of multiple *iceA* strains rather than mixed genotypes within a single strain in these cultures.

The finding that the 54 *iceA1*⁺/*iceA2*⁺ strains were mixed cultures suggested identification of both *iceA1*⁺ and *iceA2*⁺ strains from a single gastric biopsy. Fifty four (63%) of the 86 patients were therefore infected with a single *iceA* strain. Examination of the distribution of *iceA* alleles in patients with single strains demonstrated that the distribution differed between the clinical groups (χ^2 7.13, $p < 0.03$). Specifically, significantly more patients with GC (10 (67%) of 15) were infected with *iceA1* strains compared with five (23%) of 22 with gastritis alone ($p < 0.01$) (fig 6). This was not different to patients with PUD (seven (41%) of 17; $p > 0.1$).

Table 1 Protein characteristics of *iceA* variants

	JHP1132	HP27	Ca1506	1715
Variant	2B	2C	2D	2D'
Amino acids (n)	59	59	94	95
Cysteine (%)†	0	0	0	1
Proline (%)†	0	0	0	0
Topology: N terminus	"in"	"in"	"in"	"in"
Topology: C terminus	?	?	?	?
TopPred				
PTM (n)	None	None	One	One
Position‡ (score)			58–78 (0.758)	23–41 (0.615)
DAS				
PTM (n)	None	One	Two	Two
Position		32–41	37–40, 66–75	31–41, 70–76
Helix (%)†	11.7	0.0	0.0	0.0
Sheet (%)†	41.7	62.7	72.3	54.2
Other (%)†	46.7	37.3	27.7	45.8
Class†	Mixed	All-beta	All-beta	All-beta

?, not determined; Position‡, amino acid position; †from JPRED.

"in", orientation inside cell membrane; PTM, putative transmembrane segment; DAS, dense alignment sequence.

Figure 4 Topology profiles of four *iceA2* variants. Left: protein predictions from the TopPred program; right: predictions from the dense alignment sequence (DAS) program. The type strain JHP1132 (*iceA2B* variant) is indicated in the top panel and South African strains are included with the *iceA2* variants signified (2C–2D). Arrows indicate putative transmembrane regions.

Figure 5 Southern analysis of putatively clonal isolates derived from eight parent strains (lanes 1–8) that were originally *iceA1*+/2⁺ by polymerase chain reaction. Four were *iceA1*+, three *iceA2*+, and one had no hybridisation. Positive controls (lanes 9 and 10) were 26695 (*iceA1*+) and J99 (*iceA2*).

Examination of the distribution of *iceA* genotypes in all 86 patients demonstrated a significant relationship ($p < 0.05$) between infection with *iceA2* strains and patients with gastritis alone (86%) and PUD (77%) compared with patients with GC (47%).

DISTRIBUTION OF *iceA2* SUBTYPES

The size and subtype of the *iceA2* alleles were next investigated using primer set *IceA2F6*/*IceA2R5*. As previously reported,^{6,7} most isolates could be divided into two types according

Figure 6 Distribution of *iceA* genotypes in all isolates. ** $p < 0.01$ versus gastritis alone; † $p < 0.04$, †† $p < 0.003$ versus gastric cancer.

Figure 7 Distribution of *iceA2* subtypes in patients with gastritis alone or peptic ulcer disease.

to the presence of 105 nucleotide repeat sequences which resulted in PCR products of either ~229 bp (*iceA2B* or *iceA2C*) or ~334 bp (*iceA2D*). A number of isolates had either both 229 and 334 bp products or a combination of 229 bp and/or 334 bp and ~449 bp (*iceA2E*) products. Subsequent analysis of three clonally derived isolates from the latter group demonstrated only single PCR and Southern bands (fig 5), which suggested the existence of multiple *iceA2* strains, rather than multiple *iceA2* gene copies in these isolates.

Sixty five (75%) of 87 patients were infected with a single *iceA2* strain. The distribution of the *iceA2* subtypes was significantly different between the different clinical groups (χ^2 9.4, $p = 0.009$). Specifically, the *iceA2B* or *iceA2C* subtype (229 bp) was predominant in gastritis cases (26 of 35 (67%); $p < 0.02$ v PUD) (fig 7) while the *iceA2D* subtype (334 bp) was predominant in PUD cases (14 of 21 (67%)). No differences in distribution were noted among the 22 patients with multiple *iceA2* subtypes (data not shown).

COMBINATION OF *iceA*, *vacA*, AND *cagA* GENOTYPES
All strains were *cagA*⁺ and gave a single 3' amplicon. By using the method of van Doorn and colleagues,⁷ eight different combinations based on analysis of the *vacA* s region (s1 and s2), and the *iceA* type (*iceA1*, *iceA2B/C*, *iceA2D*, and *iceA2E*) were examined in all patients (fig

Figure 8 Combination analysis of *vacA/iceA* alleles and disease outcome in all clinical isolates. ** $p < 0.01$, *** $p < 0.005$ versus gastritis alone; † $p < 0.05$ versus peptic ulceration or gastric adenocarcinoma.

Table 2 Distribution of *vacA/iceA* alleles in single genotype isolates (n (%))

Allele	Gastritis (n=22)	PUD (n=17)	GC (n=15)
<i>vacAs1/iceA1</i>	2 (9)	8 (47)*	10 (67)***
<i>vacAs1/iceA2BC</i>	3 (14)	3 (18)	4 (26)
<i>vacAs1/iceA2D</i>	3 (14)	6 (35)	1 (7)
<i>vacAs2/iceA1</i>	1 (5)	0 (0)	0 (0)
<i>vacAs2/iceA2BC</i>	10 (45)	0 (0)**	0 (0)**
<i>vacAs2/iceA2D</i>	3 (14)	0 (0)	0 (0)

PUD, peptic ulcer disease; GC, gastric adenocarcinoma.

* $p < 0.01$, ** $p < 0.002$, *** $p < 0.0005$ versus gastritis.

8). Significant associations between genotypes and clinical outcome were noted (χ^2 57.8, $p < 10^{-6}$). Significantly more PUD isolates (69%; $p < 0.005$ v gastritis alone) and GC isolates (81%; $p < 0.0005$ GC v gastritis) were *vacA* s1/*iceA1*. In addition, significantly more PUD isolates were *vacA* s1/*iceA2D* (53%; $p < 0.006$ v gastritis alone). In contrast, isolates from patients with gastritis alone were identified as either *vacA* s2/*iceA1* (21%; $p < 1 \times 10^{-5}$ v PUD; $p < 0.0001$ v GC) or *vacA* s2/*iceA2* (22%; $p < 1 \times 10^{-5}$ v PUD; $p < 3 \times 10^{-5}$ v GC). In addition, a similar analysis was undertaken in patients with a single *iceA* strain, with concordant results (table 2). Significant associations between genotypes and clinical outcome were noted (χ^2 30.4, $p < 0.0008$). Significantly more PUD isolates (47%; $p < 0.01$ v gastritis) and GC isolates (67%; $p < 0.0004$ v gastritis) were *vacA* s1/*iceA1*. In contrast, significantly more ($p < 0.009$) isolates from patients with gastritis alone were *vacA* s2/*iceA2B/C*.

Discussion

These findings support the hypothesis that strain differences in *iceA* genotypes may partially explain the differences in disease outcome associated with *H. pylori* infection in South African populations.

Almost 40% of South African patients were distinguished by a high prevalence of mixed *iceA* strains while examination of cultures demonstrated that ~50% were comprised of

strains that contained different *iceA* types (either *iceA1/2* or >1 *iceA2* subtypes). The reason for the high incidence of infection with mixed *iceA* strains in African patients may simply reflect multiple colonisation but mixed strains do appear to be commonly found and range from 4% in the USA⁹ to 15% in the Netherlands,⁷ 17% in Japan,⁹ and 22% in Colombia.⁹

In the present study, analysis of *iceA* genotypes in South African patients demonstrated that although *iceA1* was more prevalent in PUD patients than in gastritis alone patients, this was not statistically significant. Previous studies in the USA⁵ and the Netherlands⁷ but not in Southeast Asia⁹ have demonstrated a strong association between this allele and PUD. It is possible that the high prevalence (~50%) of mixed *iceA* strains in PUD patients may obscure any potential relationship between the allele and the disease. There was however a strong relationship between the combination of *vacA* signal sequence subtype and *iceA* alleles, and PUD. Significantly more PUD isolates were *vacA* s1/*iceA1* compared with 40% of isolates from patients with gastritis alone.

The function of *iceA2* is unknown. In this study, amplicon size of this gene appeared to discriminate between PUD and gastritis alone. *iceA2C* (229 bp) occurred in ~62% of all isolates from South African patients with no clinically significant disease while *iceA2D* (334 bp) was more prevalent in isolates from patients with PUD. Combination *vacA/iceA* analysis demonstrated that the *vacAs1/iceA2D* was more prevalent in PUD patients (53%) than in patients with gastritis alone (23%). Consistent with these findings we have recently reported a significant relationship between the cassette structure of *iceA2* and expression in vivo; *iceA2A/B/C* mucosal transcript levels were higher than *iceA2D* levels.²¹ This suggests that downregulation of *iceA2* expression, in addition to induction of *iceA*, may also contribute to disease outcome. It is possible therefore that South African strains from patients with clinically significant disease may not readily express *iceA2* as the majority of these isolates encode the *iceA2D* variant. Alternatively, alterations in the number of repeat cassettes may result in potential changes in protein secondary structure. For example, variants 2A and 2B may exist as a single globular entity outside the cell while variants 2C and 2D are predicted to display one and two surface exposed regions, respectively. These changes may translate into differential binding and/or function of the protein.

There is a paucity of investigations of *iceA* alleles in isolates from patients with GC. In the one small study, gastric cancer isolates from Japan and Korea were distinguished by the prevalence of *iceA1* (67%) while 75% of cancer isolates from the USA were *iceA2*.⁹ The numbers in this study were relatively small which limited statistical analysis. In a second Japanese gastric cancer study, *iceA1* was associated with enhanced gastric inflammation but not adenocarcinoma per se.⁸ South African *H*

pylori cancer isolates, similar to Southeast Asian strains, were distinguished by the presence of *iceA1* and the absence of *iceA2*. In contrast however the prevalence of these alleles in cancer patients was significantly different to that found in patients without clinically significant disease in this study. In addition, gastric cancer isolates, unlike those from South African patients with PUD or gastritis, were not distinguished by any specific *iceA2* subtype. These data were strengthened when analysed in combination with the *vacA* status of the strains. Significantly more gastric cancer isolates were distinguished by *vacA* s1/*iceA1* compared with patients without disease. The putative role of IceA1 in gastric cancer is not defined but experimental studies have demonstrated that long term colonisation in both mouse and monkey models of experimental *H. pylori* infection require a wild-type *iceA1* strain.^{6, 22} In the present study, *iceA1* does not encode a restriction enzyme and probably, as has been previously suggested,¹⁴ acts as a transcriptional regulator for downstream genes. It is however possible that the higher percentage of *iceA1*⁺ strains in South African gastric cancer patients may simply reflect the observation that these patients have been potentially infected for a longer time period. Alternatively, analysis of differences in *iceA1* gene transcription and therefore protein production may reveal a functional role for this protein in this condition.¹⁴

In summary, the sequences and prevalence of *iceA1* and *iceA2* in South African *H. pylori* isolates has been investigated, the observation that ~40% of patients are infected with mixed strains made, and a novel *iceA2D* subtype identified. The potential association between *iceA1* and GC, *iceA2D* and PUD, and *iceA2C* and gastritis alone has been demonstrated in our study populations. No isolate from patients with clinically significant disease exhibited either the *vacA* s2/*iceA1* or *vacA* s2/*iceA2* allele. Overall, these data support the hypothesis that there is a difference between organisms associated with and without disease. Absolute separation cannot be made which suggests that other factors must play a role in disease pathogenesis. Analysis of *iceA* allelic types however is useful in South Africa and certain combinations of virulence factors may provide excellent negative markers for disease.

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