Secka, Ousman; Antonio, Martin; Berg, Douglas E; Tapgun, Mary; Bottomley, Christian; Thomas, Vivat; Walton, Robert; Corrah, Tumani; Thomas, Julian E; Adegbola, Richard A; (2011) Mixed infection with cagA positive and cagA negative strains of Helicobacter pylori lowers disease burden in The Gambia. PloS one, 6 (11). e27954-. ISSN 1932-6203 DOI: https://doi.org/10.1371/journal.pone.0027954

Downloaded from: http://researchonline.lshtm.ac.uk/id/eprint/27195/

DOI: https://doi.org/10.1371/journal.pone.0027954

Usage Guidelines:

Please refer to usage guidelines at https://researchonline.lshtm.ac.uk/policies.html or alternatively contact researchonline@lshtm.ac.uk.

Available under license: http://creativecommons.org/licenses/by/2.5/
Mixed Infection with \textit{cagA} Positive and \textit{cagA} Negative Strains of \textit{Helicobacter pylori} Lowers Disease Burden in The Gambia

Ousman Secka\textsuperscript{1}*\textsuperscript{,} Martin Antonio\textsuperscript{1}, Douglas E. Berg\textsuperscript{2}, Mary Tapgun\textsuperscript{1}, Christian Bottomley\textsuperscript{4}, Vivat Thomas\textsuperscript{1}, Robert Walton\textsuperscript{1}, Tumani Corrah\textsuperscript{1}, Julian E. Thomas\textsuperscript{2}, Richard A. Adegbola\textsuperscript{1}\textsuperscript{*}

\textsuperscript{1}Medical Research Council Unit, Fajara, The Gambia, \textsuperscript{2}School of Clinical Medical Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom, \textsuperscript{3}Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, United States of America, \textsuperscript{4}Medical Research Council, Tropical Epidemiology Group, London School of Hygiene and Tropical Medicine, London, United Kingdom

Abstract

\textbf{Background:} The prevalence of \textit{Helicobacter pylori} including strains with putatively virulent genotypes is high, whereas the \textit{H. pylori}-associated disease burden is low, in Africa compared to developed countries. In this study, we investigated the prevalence of virulence-related \textit{H. pylori} genotypes and their association with gastroduodenal diseases in The Gambia.

\textbf{Methods and Findings:} DNA extracted from biopsies and \textit{H. pylori} cultures from 169 subjects with abdominal pain, dyspepsia or other gastroduodenal diseases were tested by PCR for \textit{H. pylori}. The \textit{H. pylori} positive samples were further tested for the \textit{cagA} oncogene and \textit{vacA} toxin gene. One hundred and twenty one subjects (71.6\%) were \textit{H. pylori} positive. The \textit{cagA} gene and more toxigenic \textit{s1} and \textit{m1} alleles of the \textit{vacA} gene were found in 61.2\%, 76.9\% and 45.5\% respectively of Gambian patients harbouring \textit{H. pylori}. There was a high prevalence of \textit{cagA} positive strains in patients with overt gastric diseases than those with non-ulcerative dyspepsia (NUD) (p = 0.05); however, mixed infection by \textit{cagA} positive and \textit{cagA} negative strains was more common in patients with NUD compared to patients with gastric disease (24.5\% versus 0\%; p = 0.002).

\textbf{Conclusion:} This study shows that the prevalence of \textit{H. pylori} is high in dyspeptic patients in The Gambia and that many strains are of the putatively more virulent \textit{cagA}\textsuperscript{+}, \textit{vacAs1} and \textit{vacAm1} genotypes. This study has also shown significantly lower disease burden in Gambians infected with a mixture of \textit{cagA}-positive and \textit{cagA}-negative strains, relative to those containing only \textit{cagA}-positive or only \textit{cagA}-negative strains, which suggests that harbouring both \textit{cagA}-positive and \textit{cagA}-negative strains is protective.

Introduction

\textit{Helicobacter pylori} is a genetically diverse microaerophilic gram negative bacterial species that chronically infects the human gastric mucosa, often starting in infancy and lasting for life \cite{1}. About 50\% of the world’s adult population is colonized, with prevalences of over 80\% in many developing countries including The Gambia \cite{2,3,4}. Earlier reports indicated high prevalence of \textit{H. pylori} colonization, but a low frequency of \textit{H. pylori}-associated disease in Africa \cite{2,5,6}, a phenomenon that was called the “African enigma” \cite{2}. DNA sequencing of housekeeping and virulence genes has shown that different sets of genotypes predominate in different human populations \cite{7}. Of particular interests have been \textit{H. pylori’s} \textit{cagA} oncogene and toxigenic \textit{s1} and \textit{m1} alleles of its \textit{vacA} gene, which have been implicated in gastroduodenal diseases caused by this pathogen both in epidemiologic \cite{8,9}, experimental animal and cell culture infection \cite{10} studies. This said, several studies from different world regions have not detected such an association \cite{8,11,12}, an outcome suggesting the possibility of other virulence-modulating factors.

Individuals can be colonized by either a single or multiple strains of \textit{H. pylori}, and even colonization by what is initially a single strain can, over time, lead to the emergence of multiple \textit{H. pylori} subpopulations, due variably to mutation or to genetic recombination either between duplicate sequences in the single strain’s genome or with DNAs from other transiently colonizing strains \cite{8}. The prevalence of such mixed infections has been reported to vary (5-68\%) \cite{13,14,15,16,17} depending on geographical region, whether in a developed or developing country (low and high overall infection risk, respectively), and probably also methods of analysis. The \textit{H. pylori} virulence-associated vacuolating cytotoxin (\textit{vacA}) and \textit{cag} pathogenicity island (\textit{cag PAI}) genes, and
also the cag empty site in strains lacking the cag PAI, are typically found in only one copy per genome [17–20]. Accordingly, detection of both the cagA gene and the cag empty site, or of both s1 and s2 (signal sequence; at 5’ end of gene) or both m1 and m2 (middle region) alleles of vacA in a biopsy or in pool of H. pylori from a person indicates mixed infection.

We wondered if having mixed infection might influence the risk of gastric disease; for example, if strains of different genotypes might occupy a broader range of niches in the stomach as has been seen during experimental infection [21] and thereby impact on clinical outcome. In this study, we investigated the genotypes of H. pylori in The Gambia and the relation of apparently single versus mixed infections to gastroduodenal diseases.

Materials and Methods

Ethics statement

Ethical approval of this study was obtained from the joint Medical Research Council (MRC) Unit, The Gambia/Gambia Government Ethics Committee and Division of Microbiology Infectious Diseases (DMID) International Review Board.

Patients

Clinical data from the MRC Unit in The Gambia revealed that of 428 patients with gastric complaints investigated by gastric endoscopy between 2003–2008, 8 (1.9%) had gastric carcinoma, 20 (4.7%) and 15 (3.5%) had gastric and duodenal ulcers respectively, and that the others (89.9%) did not have such overt disease (diagnosed as non-ulcer dyspepsia; NUD (data not shown). All patients referred for endoscopy to the MRC Unit during the years 2003 to 2008 were initially considered eligible for inclusion: All 169 subjects who agreed to join this study provided written informed consent; in addition, for children less than 10 years, antral biopsies were obtained only after informed written parental consent. Patients with severe oesophago-gastroduodenal disease, including those with gastro-oesophageal varices, a small number with advanced gastric cancer and those on H. pylori eradication therapy, were excluded from the study. One hundred and twenty one patients from whose biopsies we successfully amplified virulence genes were analysed here. The mean age of these subjects was 35, ranging from 9 to 80 years. All the subjects were Gambians and most of them (75) came from the Greater Banjul Area, 38 from the West Coast region, 5 from Lower River region and 3 from North Bank region of The Gambia.

Gastroscopy results

Endoscopic examination showed that of the 121 study subjects, 11 had gastric ulcer, 7 had duodenal ulcer, 1 had both gastric and duodenal ulcers, 7 had gastric erosions, 1 had gastric carcinoma and all other subjects (94) who presented with either abdominal pain or dyspepsia had no evidence or history of gastric or duodenal ulcers.

The biopsies collected from patients were stored in Brain Heart Infusion (BHI) broth containing 20% glycerol and transported on ice to the laboratory for processing or stored at −70°C until used. Our previous data demonstrated that in The Gambia detection of mixed H. pylori strains in individual biopsies was best undertaken by PCR amplification directly from biopsy material rather than by bacterial culture [22].

Genomic DNA extraction directly from biopsies

Total genomic DNA was extracted directly from the biopsy material by using a combination of bead-beater and the QIAamp DNA isolation kit (Qiagen, UK) as previously described [22].

PCR amplification

PCR was performed to detect the cagA gene and cag empty site, and the signal sequence (s1 and s2) and middle region (m1 and m2) alleles of the vacA gene, as previously described [23] using the primers listed in table 1 and the following cycling conditions: 30 cycles of 94°C for 1 min, 55°C or 60°C for 1 min and 72°C for 1 min. The amplified genes were detected by electrophoresis in a 1.5% gel with ethidium bromide (500 ng/ml) and bands were visualized using Gel Doc 2000 (Bio-Rad laboratories, Milan, Italy). The presence of a particular gene or allele was inferred when a product of the expected size (table 1) was obtained using appropriate primers.

Statistical analysis

We assessed the prevalences of infection with single vs. multiple strains. For the cagA gene, for example, we noted the occurrence of cagA positive, cag empty site and mixed (cagA positive and cag PAI negative) infections. Prevalences were compared between disease groups and p-values were determined using Fisher’s exact test.

| Table 2. Prevalence of Helicobacter pylori genotypes. |
|--------------------------------------------------------|
| H. pylori genotypes | n  | %  |
|---------------------|----|----|
| cagA/+                | 74 | 61.2 |
| cagA−                 | 21 | 17.4 |
| cagA+ & cagA−         | 23 | 19.0 |
| No amplification of cagA or cag empty site          | 3  | 2.5 |
| s1                   | 93 | 76.9 |
| s2                   | 23 | 19.0 |
| s1 & s2              | 1  | 0.8 |
| no amplification of s1 or s2                         | 4  | 3.3 |
| m1                   | 55 | 45.5 |
| m2                   | 36 | 29.8 |
| m1 & m2             | 22 | 18.2 |
| No amplification of m1 or m2                         | 8  | 6.6 |

doi:10.1371/journal.pone.0027954.t002

Table 1. Primers used in this study.

| Region      | Primer | Nucleotide sequence | bp   | reference |
|-------------|--------|---------------------|------|-----------|
| cagA        | cagAF  | gat aac agg caa gct ttt gag g | 349  | [23]      |
|             | cagAR  | cagA<sup>+</sup>     |      |           |
| cag empty   | Uni-1  | aca ttt tgg cta aat aaa cgc tg | 535  | [23]      |
| site        | R5280  | cgg gtt cag acg cat ttt ccc tta atc | 535  | [23]      |
| vacAs1      | Va1-F  | atg gaa ata caa caa aca cac ctt ctg ggc gaa c | s1 | 259      |
| vacAs2      | Va1-R  | ggt cca aat ggc gtg atg g | s2  | 289      |
| vacAm1      | Va3-F  | ggt cca aat ggc gtg atg g |     |           |
| vacAm2      | Va3-R  | cca ttt gta cct gta gaa ac |     |           |
|             | Va4-R  | gga gcc cca gga aac att g | 352  | [23]      |
|             | Va4-F  | cat aac tag cgc ctt gca c |     |           |

doi:10.1371/journal.pone.0027954.t001
Effect of Mixed Infection of *H. pylori* on Disease

### Results

**Prevalence of *H. pylori* genotypes**

One hundred and twenty one patients of the 169 study participants were inferred to be infected with *H. pylori* when DNAs extracted from their biopsies were tested by PCR for the presence of *H. pylori* cagA gene and cag empty site. Seventy four biopsies (61.2%) were positive for the cagA gene only, 21 (17.4%) were positive for the cag empty site only and 23 (19%) were positive for both. In parallel we also tested for the cagA gene presence and allele types. In all, 93 of 121 (76.9%) were positive only for the cagA allele, 23 (19.0%) were positive only for the vacA2 allele and 1 (0.8%) was positive for both. Only m1 or only m2 alleles of vacA were detected in 55 (45.5%) and 36 (29.8%) of biopsies tested respectively; both m1 and m2 (mixed infections) were found in 22 (18.2%) biopsies and up to 6.6% of biopsy DNAs failed to amplify for individual alleles (table 2).

**Association between *H. pylori* genotypes**

Of the 93 *H. pylori* strains that were positive only for vacA1, 72 (77.4%) were cagA positive compared with only 1 (4.3%) cagA positive among the 23 strains that were positive only for vacA2; most (16) of them contained the cag empty site allele only (table 3). Similarly, nearly all s1m1 positive biopsies (92.5%) contained cagA genes, whereas none of those containing only vacA s2m2 allele were cagA positive (table 3).

**Association of *H. pylori* virulence genes with upper gastric diseases**

cagA positive *H. pylori* strains were found more frequently among study participants with gastroduodenal diseases than those with NUD: duodenal ulcers (6/7; 85.7%), gastric erosions (5/7, 71.4%), gastric ulcers (8/11, 72.7%); no overt gastric disease (53/94, 56.4%) (table 4). In the 27 patients with overt gastric disease, 77.8% were cagA positive compared to 56.4% of those with NUD (p-value = 0.05, table 5). Toxigenic s1m1 alleles were found in 6 of the 11 (54.5%) patients diagnosed with gastric ulcer, 42.9%, 42.9% and 42.6% in those with duodenal ulcers, gastric erosions and NUD, respectively (table 6). The prevalence of vacA alleles were similar in the two groups of patients; overt disease vs. NUD. That is, no association was found between vacA alleles and clinical outcome (p = 0.94, table 7).

All 27 subjects with overt gastric diseases were of uniform cagA status (that is, uniquely cagA gene positive or cag empty site positive), whereas only 72.3% (68/94) of NUD were of uniform status; the other 23 contained mixed (cagA positive, cag empty site positive) infections. Three other biopsy samples did not give cagA gene or cag empty site amplification (table 5). This association between uniform cagA status and overt disease was statistically significant (p = 0.002).

In terms of age distribution, no association was found between age and overt gastric disease (24.5%<30 years, 12.5% 30–40 years and 27.8%>40 years; p-value = 0.26, table 8), or frequency of

### Table 3. Association of vacA with cagA *Helicobacter pylori* genotypes.

| *H. pylori* genotypes | cagA<sup>+</sup> | cagA<sup>−</sup> | cagA<sup>+&</sup>cagA<sup>−</sup> | Incomplete cagA | Total |
|-----------------------|----------------|----------------|----------------|-----------------|-------|
| s1m1                  | 49 (92.5)      | 1 (1.9)        | 3 (5.7)        | 0 (0)           | 53    |
| s1m2                  | 9 (50)         | 3 (16.7)       | 5 (27.8)       | 1 (5.6)         | 18    |
| s2m1                  | 0 (0)          | 16 (88.9)      | 2 (11.1)       | 0 (0)           | 18    |
| s2m2                  | 0 (0)          | 0 (0)          | 0 (0)          | 0 (0)           | 0     |
| s1m1m2                | 12 (66.7)      | 6 (33.3)       | 0 (0)          | 0 (0)           | 18    |
| s1s2m1m2              | 0 (0)          | 0 (0)          | 1 (100)        | 0 (0)           | 1     |
| s2m1m2                | 0 (0)          | 0 (0)          | 3 (100)        | 0 (0)           | 3     |
| Incomplete VacA       | 4 (40)         | 1 (10)         | 3 (30)         | 2 (20)          | 10    |

Incomplete cagA = cagA and cag empty site were not detected.
Incomplete vacA = either vacA s or vacA m regions were not detected (4/10 vacAs1 was detected & vacAm was missing, 2/10 vacAs2 detected and vacAm missing, 2/10 vacAm1 detected and vacAs missing and for 2/10 both vacAs and vacAm were missing).

### Table 4. Association between cagA genotypes and disease type.

|          | DU (n=74) | GC (n=7) | GE (n=5) | GU (n=8) | GUDU (n=1) | NUD (n=53) | Total (n=94) |
|----------|-----------|----------|----------|----------|------------|------------|-------------|
| cagA<sup>+</sup> | 6 (85.7)  | 1 (100)  | 5 (71.4) | 8 (72.7)  | 1 (100)    | 53 (56.4)  | 74 (61.2)   |
| cagA<sup>−</sup> | 1 (14.3)  | 0 (0)    | 2 (28.6) | 3 (27.3)  | 0 (0)      | 15 (16.0)  | 21 (17.4)   |
| cagA<sup>+</sup>& cagA<sup>−</sup> | 0 (0)    | 0 (0)    | 0 (0)    | 0 (0)    | 0 (0)      | 23 (24.5)  | 23 (19.0)   |
| No amplification | 0 (0)    | 0 (0)    | 0 (0)    | 0 (0)    | 0 (0)      | 3 (3.2)    | 3 (2.5)     |
| Total | 7 (5.8)   | 1 (0.8)  | 7 (5.8)  | 11 (9.1) | 1 (0.8)    | 94 (77.7)  | 121 (100)   |

DU = duodenal ulcer, GC = gastric carcinoma, GE = gastric erosion, GU = gastric ulcer, GUDU = gastric ulcer and duodenal ulcer, NUD = Non-ulcerative disease.

doi:10.1371/journal.pone.0027954.t003

doi:10.1371/journal.pone.0027954.t004
mixed infection (15.1% < 30 years, 18.8% 30–40 years, 25.5% > 40 years; p-value = 0.46, table 9).

Discussion

*H. pylori* infection is common in dyspeptic adults in The Gambia [4,22], as is typical of developing countries. The range of *H. pylori* genotypes implicated in overt gastroduodenal disease as opposed to benign colonization or possibly even beneficial carriage [9,24] had not been extensively investigated in Sub–Saharan Africa. Here we studied the distribution of *H. pylori*’s main virulence genes, *cagA* and toxigenic alleles of *vacA*, in The Gambia, and their possible associations with disease outcome.

The prevalence of gastroduodenal disease (10%) that we detected endoscopically is similar to that reported elsewhere in Sub–Saharan Africa. We found that just over half of disease among people with gastric complaints of sufficient severity were missing (2/10 vacA s was detected & vacA m missing, 2/10 vacAm2 detected and vacAs2 missing and for 2/10 both vacAs and vacAm were missing). doi:10.1371/journal.pone.0027954.t007

| Table 5. cagA and clinical outcome. |
|------------------------------------|
| cagA status | Overt gastric disease | NUD |
|            | n (%) | |
| cagA*      | 21 77.8 | 53 56.4 |
| cagA* & cagA+ | 6 22.2 | 15 16.0 |
| no amplification | 0 0 | 23 24.5 |
| Total       | 27 100 | 94 100 |

doi:10.1371/journal.pone.0027954.t005

Table 6. Association between vacA genotypes and disease type.

| vacA status | DU* | GC* | GE* | GU* | GUDU* | NUD* | Total |
|-------------|-----|-----|-----|-----|-------|------|-------|
| s1m1        | 3 (42.9) | 1 (100) | 3 (42.9) | 6 (54.5) | 0 (0) | 40 (42.6) | 53 (43.8) |
| s1m2        | 1 (14.3) | 0 (0) | 2 (28.6) | 0 (0) | 0 (0) | 15 (16.0) | 18 (14.9) |
| s2m2        | 0 (0) | 0 (0) | 2 (28.6) | 3 (27.3) | 0 (0) | 13 (13.8) | 18 (14.9) |
| s1m1m2      | 3 (42.9) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (1.1) | 8 (0.8) |
| s1s2m1m2    | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 3 (3.2) | 3 (2.5) |
| Incomplete 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (1.1) | 8 (0.8) |
| Total       7 | 1 | 7 | 1 | 11 | 1 | 94 | 121 |

*Du = Duodenal ulcer; GC = gastric cancer; GE = gastric erosion; GU = gastric ulcer; GUDU = gastric and duodenal ulcers; NUD = non-ulcerative diseases.
Incomplete vacA = either vacA s or vacA m regions were not detected (4/10 vacAs1 was detected & vacAm was missing, 2/10 vacAm1 detected and vacAs2 missing and for 2/10 both vacAs and vacAm were missing).

Table 7. Association between vacA genotypes and clinical outcome.

| vacA genotypes | Overt gastric disease | NUD |
|----------------|-----------------------|-----|
|                | n (%) | n (%) |
| s1m1           | 13 48.1 | 40 42.5 |
| s1m2           | 3 11.1 | 15 16.0 |
| s2m2           | 5 18.5 | 13 13.8 |
| s1m1m2         | 4 14.8 | 14 14.9 |
| s1s2m1m2       | 0 0 | 1 1.1 |
| s2m1m2         | 0 0 | 3 3.2 |
| Incomplete vacA | 2 7.4 | 8 8.5 |
| Total          | 27 100 | 94 100 |

Incomplete vacA = either vacA s or vacA m regions were not detected (4/10 vacAs1 was detected & vacAm was missing, 2/10 vacAm1 detected and vacAs2 missing and for 2/10 both vacAs and vacAm were missing).

Table 8. Association between age and disease.

| Age groups | Overt disease | NUD |
|------------|--------------|-----|
|            | n (%) | n (%) | Total |
| <30 years  | 13 (24.5) | 40 (74.5) | 53 |
| 30–40 years | 4 (12.5) | 28 (87.5) | 32 |
| >40 years  | 10 (27.8) | 26 (72.2) | 36 |

p-value = 0.26. doi:10.1371/journal.pone.0027954.t008
Evidence for the intimate association between humans and Helicobacter pylori Nature 445: 915–916.
8. Blaser MJ, Berg DE (2001) Helicobacter pylori genetic diversity and risk of human disease. J Clin Invest 107: 767–773.
9. Sugimoto M, Yamaoka Y (2009) The association of vacA genotype and Helicobacter pylori-related disease in Latin American and African populations. Clin Microbiol Infect 15: 835–842.
10. Rieder G, Fischer W, Haas R (2005) Interaction of Helicobacter pylori with host cells: function of secreted and translocated molecules. Curr Opin Microbiol 8: 67–73.
11. Agah A, Graham DY (2005) Evidence-based examination of the African enigma in relation to Helicobacter pylori infection. Scand J Gastroenterol 40: 523–529.
12. Graham DY, Lu H, Yamaoka Y (2009) African, Asian or Indian enigma, the East Asian Helicobacter pylori: facts or medical myths. J Dig Dis 10: 77–84.
Effect of Mixed Infection of *H. pylori* on Disease

13. Boyanova L, Markovska R, Yordanov D, Marina M, Ivanova K, et al. (2009) High prevalence of virulent *Helicobacter pylori* strains in symptomatic Bulgarian patients. Diag Microbiol Infect Dis 64: 374–380.

14. Kim YS, Kim N, Kim JM, Kim MS, Park JH, et al. (2009) *Helicobacter pylori* genotyping findings from multiple cultured isolates and mucosal biopsy specimens: strain diversities of *Helicobacter pylori* isolates in individual hosts. Eur J Gastroenterol & Hepatology 21: 522–528.

15. Matteo MJ, Granados G, Pérez CV, Olmos M, Sanchez C, et al. (2007) *Helicobacter pylori* cag pathogenicity island genotype diversity within the gastric niche of a single host. J Med Microbiol 56: 664–669.

16. Miernyk K, Morris J, Bruden D, McMahon B, Hurlburt D, et al. (2011) *Helicobacter pylori* vacA genotypes and vacA/cag genotypes among Alaskan and Their Correlation with Clinical Disease. J Clin Microbiol 49: 3114–3121.

17. Monynalev K, Smirnova O, Govorun V (2003) *Helicobacter pylori* Genotypes in Russia. Eur J Clin Microbiol Infect Dis 22: 573–574.

18. Ahmed N, Sechi L (2005) *Helicobacter pylori* and gastrointestinal pathology: new threats of the old friend. Ann Clin Microbiol Antimicrob 4: 1.

19. Owen RJ, Hurtado A, Banavala N, Abd Y, Davies GR, et al. (1994) Conservation of the cytotoxin-associated (cagA) gene of *Helicobacter pylori* and investigation of association with vacuolating cytotoxin activity and gastroduodenal disease. FEMS Immunol Med Microbiol 9: 307–315.

20. van Doorn L, Figueiredo C, Sanna R, Plaisier A, Schneeberger P, et al. (1998) Conservation of the cytotoxin-associated (cagA) and vacA/cag genotypes and coinfection vacA genotypes in Nigerian patients with duodenal ulcer disease. J Med Microbiol 51: 851–834.

21. Tegtmeyer N, Zabler D, Schmidt D, Hartig R, Brandt S, et al. (2009) Importance of EGFR receptor, HER2/Neu and Erk1/2 kinase signalling for host cell elongation and scattering induced by the *Helicobacter pylori* CagA protein: antagonistic effects of the vacuolating cytotoxin. Cell Microbiol 11: 480–505.

22. Alam SI, Bansod S, Singh L (2008) Immunization against *Clostridium perfringens* cells elicits protection against *Clostridium tetani* in mouse model: identification of cross-reactive proteins using proteomic methodologies. BMC Microbiology 8: 194.

23. Berg S, Treilbøn B, Persson E, Backhaus E, Larsson P, et al. (2006) Serotypes of *Streptococcus pneumoniae* isolated from blood and cerebrospinal fluid related to vaccine serotypes and to clinical characteristics. Scand J Infect Dis 38: 427–432.

24. Panangala VS, Shoemaker CA, Klessis PH, Mitra A, Kusso R (2009) Cross-protection elicited in channel catfish (*Ictalurus punctatus* Rafinesque) immunized with a low dose of virulent *Edwardsiella ictaluri* strains. Aquaculture Research 40: 915–926.

25. Muthukopahiyay AK, Kersulity D, Datta S, Ito Y, Chowdhury A, et al. (2002) Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta. J Bacteriol 184: 3219–27.

26. Mukhopadhyay AK, Kersulyte D, Datta S, Ito Y, Chowdhury A, et al. (2002) Clinical Relevance of the cagA, vacA, and iceA Status of *Helicobacter pylori*. Gastroenterol 115: 50–66.

27. Yamaoka Y, Kato M, Asaka M (2008) Geographic differences in gastric cancer incidence can be explained by differences between *Helicobacter pylori* strains. Intern Med 47: 1077–1085.

28. Nguyen LT, Uchida T, Murakami K, Fujikoa T, Moriyama M (2008) *Helicobacter pylori* virulence and the diversity of gastric cancer in Asia. J Med Microbiol 57: 1445–53.

29. Palacios G, Hornig M, Cisterna D, Savji N, Bussetti AV, et al. (2009) Importance of *EGF* receptor, HER2/Neu and Erk1/2 kinase signalling for host cell elongation and scattering induced by the *Helicobacter pylori* CagA protein: antagonistic effects of the vacuolating cytotoxin. Cell Microbiol 11: 480–505.

30. van Doorn L, Figueiredo C, Sanna R, Plaisier A, Schneeberger P, et al. (1998) Conservation of the cytotoxin-associated (cagA) and vacA/cag genotypes in Nigerian patients with duodenal ulcer disease. J Med Microbiol 51: 851–834.

31. Tegtmeyer N, Zabler D, Schmidt D, Hartig R, Brandt S, et al. (2009) Importance of EGFR receptor, HER2/Neu and Erk1/2 kinase signalling for host cell elongation and scattering induced by the *Helicobacter pylori* CagA protein: antagonistic effects of the vacuolating cytotoxin. Cell Microbiol 11: 480–505.

32. Alam SI, Bansod S, Singh L (2008) Immunization against *Clostridium perfringens* cells elicits protection against *Clostridium tetani* in mouse model: identification of cross-reactive proteins using proteomic methodologies. BMC Microbiology 8: 194.

33. Berg S, Treilbøn B, Persson E, Backhaus E, Larsson P, et al. (2006) Serotypes of *Streptococcus pneumoniae* isolated from blood and cerebrospinal fluid related to vaccine serotypes and to clinical characteristics. Scand J Infect Dis 38: 427–432.

34. Panangala VS, Shoemaker CA, Klessis PH, Mitra A, Kusso R (2009) Cross-protection elicited in channel catfish (*Ictalurus punctatus* Rafinesque) immunized with a low dose of virulent *Edwardsiella ictaluri* strains. Aquaculture Research 40: 915–926.

35. Muthukopahiyay AK, Kersulity D, Datta S, Ito Y, Chowdhury A, et al. (2002) Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta. J Bacteriol 184: 3219–27.

36. Yamaoka Y, Kato M, Asaka M (2008) Geographic differences in gastric cancer incidence can be explained by differences between *Helicobacter pylori* strains. Intern Med 47: 1077–1085.

37. Segal I, Ally R, Mitchell H (2001) *Helicobacter pylori*: an African perspective. Q J Med 94: 561–565.

38. Chen XJ, Yan J, Shen YF (2005) Dominant cagA/vacA genotypes and coinfection frequency of *H. pylori* in peptic ulcer or chronic gastritis patients in Zhejiang province and correlations among different genotypes, coinfection and severity of the diseases. Chin Med J (Engl) 20: 460–467.

39. Kumar S, Kumar A, Dixit VK (2010) Diversity in the cag pathogenicity island of *Helicobacter pylori* isolates in populations from North and South India. J Med Microbiol 59: 32–40.

40. Nguyen LT, Uchida T, Murakami K, Fujikoa T, Moriyama M (2008) *Helicobacter pylori* virulence and the diversity of gastric cancer in Asia. J Med Microbiol 57: 1445–53.

41. Palacios G, Hornig M, Cisterna D, Savji N, Bussetti AV, et al. (2009) Importance of *EGF* receptor, HER2/Neu and Erk1/2 kinase signalling for host cell elongation and scattering induced by the *Helicobacter pylori* CagA protein: antagonistic effects of the vacuolating cytotoxin. Cell Microbiol 11: 480–505.