Prevalence of $cagE$ in *Aggregatibacter actinomycetemcomitans*

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ABSTRACT

Aggregatibacter actinomycetemcomitans is a gram-negative bacterium that has been shown to be a risk indicator in the development of aggressive periodontitis. In some A. actinomycetemcomitans strains a gene named cagE has been found. This gene is not well studied in A. actinomycetemcomitans but a similar gene, a homologue, has been found in Helicobacter pylori, where it was shown to be a virulence factor that aggravates development of ulcers. The aim of this study was to investigate whether the presence of the cagE-gene could serve as a marker for virulent A. actinomycetemcomitans strains, strongly promoting the development of periodontal disease. To examine this, 244 A. actinomycetemcomitans strains collected from a study cohort of school children in Ghana were analysed by PCR and electrophoresis to see which possessed the cagE gene. The results of the PCR analysis were then compared with previously collected clinical data, linked to the samples, regarding attachment loss and progression of attachment loss. 23 of the strains were found to have the cagE-gene but no correlation was found between the carriage of the gene and attachment loss. There was however a statistically significant relationship between the presence of cagE and progression of attachment loss. We have therefore concluded that A. actinomycetemcomitans strains with possession of cagE might be of a more aggressive character, and that the gene in the future possibly could be used as a diagnostic marker to predict a more rapid pattern of the progression of periodontal disease in an individual.
INTRODUCTION

Periodontal disease is one of the most widespread diseases across the globe. The disease manifests itself in the form of loss of alveolar support, which may eventually lead to loss of teeth affected (Pihlstrom et al., 2005). It is now known that the etiology of the disease includes the bacterial biofilm, plaque, which constantly forms on the tooth surfaces. Dental bacterial plaque give rise to an inflammatory process in the tissues around the tooth surface and if this inflammation continues it may lead to a degeneration of the tissue and eventually a reduced attachment level around the affected teeth (Derveau, 2010; Kinane et al., 2006).

There are different forms of periodontitis. There is the chronic periodontitis, which is characterized by a lack of oral hygiene and shows a great amount of plaque and calculus. It generally affects adults and elderly and it has a relatively slow progress (Pihlstrom et al., 2005, Derveau, 2010). According to Swedish Agency for Health Technology Assessment and Assessment of Social Services, up to 40 % of the adult population is estimated to suffer from chronic periodontitis, with any form of attachment loss. Apart from the chronic, there is the aggressive form of periodontitis. This is characterized by a very rapid attachment loss affecting to higher extent than chronic periodontitis younger people. In this case the amount of plaque and calculus that is seen is not in proportion to the intense inflammation responses that occur. One can also see a variety of disease prevalence between ethnic groups and geographic regions where areas such as Asia and Africa have a greater prevalence of the condition (Kassebaum et al., 2014).

In aggressive periodontitis, as in the case even in chronic periodontitis, it has been shown that it is not only the amount of bacteria in plaque that matters but also the species composition (Van Winkelhoff et al., 2002). Some bacteria appear to play a major role when it comes to initiating the powerful immune response that causes rapid disease progression. One of these organisms is Aggregatibacter actinomyctemcomitans (Fine et al., 2006). A. actinomyctemcomitans is a gram negative rod-shaped bacterium that produces several virulence factors which facilitate infection and survival in the human body. Examples of these factors are leukotoxin, which kills immune cells such as monocytes and granulocytes, and cytolethal distending toxin, which may cause human cells to go into apoptosis (Henderson et al., 2010).

Large genetic variation exists within A. actinomyctemcomitans. In a polygenetic analysis (Kaplan et al., 2002) a great diversity within the species was found. Based on serotype, 16S
rRNA type, and LKT, flp-1, CDT, and AP-PCR genotypes, the species were divided into three clusters. Cluster one contains serotype b, cluster two serotype c, and cluster three serotypes a, d, e and f (Kaplan et al., 2002). The leukotoxicity differs between the different serotypes of *A. actinomycetemcomitans* and in particular one genotype, within the serotype b, called JP2 appears to be particularly virulent, expressing high levels of leukotoxin (Åberg et al., 2014a). The characteristic for the *A. actinomycetemcomitans* JP2 genotype is that it has a deletion of several base pairs in the leukotoxin promoter region (Claesson et al., 2015; Brogan et al., 1994).

One of the genes found in the genome of some strains of *A. actinomycetemcomitans* is the so-called "cytotoxin-associated gene E" (cagE) (Teng and Hu, 2003; Kittichotirat et al., 2011). This gene is little studied in *A. actinomycetemcomitans* and therefore it is not fully known whether its gene product serves any function in virulence. What is known is that a protein homologous to cagE with a 39% amino acid similarity (Teng and Hu, 2003) plays an important role in virulence of the bacterium *Helicobacter pylori*. Expression of cagE was associated with elevated levels of interleukin-8 in AGS and KATO-III gastric cell lines, a cytokine that is known to aggravate the inflammation process. Studies indicate that ulcerative duodenitis more often develop in people infected with cagE positive isolates of *H. pylori* (Day et al., 2000). This indicates that the presence of the cagE gene in *H. pylori* can serve as a marker to determine if the infection is critical for the development of gastric mucosal ulcers. On basis of such knowledge, the cagE gene might be helpful in the process of identifying virulent clones of the bacterium, and make it possible to treat people affected accordingly. As cagE can be found in several different *A. actinomycetemcomitans* isolates (Teng and Hu, 2003), our goal with this study was to investigate whether cagE could serve as a marker for highly virulent strains, associated with the development of periodontal disease. In a previous study (Teng and Zhang, 2005) it is implied that the CagE-protein in *A. actinomycetemcomitans* could have a possible apoptotic effect on human epithelial cells (Teng and Zhang, 2005). For this cause we have analysed a collection of 249 *A. actinomycetemcomitans* isolates from school children in Ghana, collected for a PhD thesis work made by Carola Höglund Åberg (Åberg et al., 2012).

We examined, by PCR and electrophoresis, which of these strains that were carrying the cagE gene. The information collected was then compared with the clinical data from the Ghana study (Åberg et al., 2012), to investigate if there was a statistical significance between the
prevalence of cagE and clinical manifestations of the disease. Based on our findings, we concluded that the cagE gene could serve as a gene marker for virulent A. actinomycetemcomitans strains, and through more research perhaps help predict a development of periodontal disease and attachment loss.

MATERIALS AND METHODS

Bacterial strains and Study population
The bacterial strains, which this paper is based on consists of 249 collected A. actinomycetemcomitans strains from a cohort study of children and adolescents in Ghana who were followed for two years (Åberg et al., 2012). The age ranged from between 10 and 19 years old with an average age of 13.2. The bacterial samples were acquired with ethical permit and the patients’ informed consent. Information regarding the general health, possible antibiotic treatment, previous dental care, tobacco use and oral hygiene were collected (Åberg et al., 2012).

Clinical data
In this work, we utilized registered data on clinical variables such as probing pocket depth and the distance between the free gingival margin and the enamel cement border (Åberg et al., 2012). This was done at the distal and mesial surfaces. At the follow-up, two years later, the tests were conducted under the same procedure. Attachment loss was recorded as the difference distance from gingival margin and enamel cement border, and the measured pocket depth.

PCR analysis
DNA templates for PCR analysis of the 249 A. actinomycetemcomitans isolates were earlier prepared by boiling a few colonies from agar cultures in H2O for 5 minutes. The templates were then stored in the freezer until use.

In a PCR reaction, 2 µl of template was added along with 23 ul of "master mix" containing 22 µl H2O and 1 µl forward primer (5'-GGATCCGTCCTGAAAATTTTATTAGCTTG-3'), and reverse primer (5'- CTGCAGTTAAACGACCTTTAAACATTTTTTTA-3') (Teng and Hu, 2003) into tubes of “Pure Taq Ready-To-Go PCR Beads” (GE Healthcare). The mixture was then subject to PCR using these cycling conditions: 94 °C for 1 min followed by 9 °C for 30
sec, 56 °C for 30 sec and 72 °C for 1 min repeated 34 times followed by 72 °C for 7 min. When finished, 10 µl of loading buffer was added to each sample. When electrophoresis was carried out, 10 µl of PCR reaction mixture with loading buffer was loaded on 1 % agarose gels containing 1 × GelRed Nucleic Acid Stain (Biotium). To identify the PCR product corresponding to cagE, a DNA size marker (Invitrogen, Carlsbad, Ca, USA) was also loaded on the gels. As a positive control in the PCR reactions a DNA from a JP2 strain named HK 1651 was used, and H2O was used as negative control. The samples were run under 98 mA and 123 volts in about 1 hour. The agarose gel was then transferred to a UV cabinet equipped with a camera where the photo was taken. A sample was considered cagE-positive if a strong band at the size 1000 bp could be detected.

Literature
Literature for this study were found using the PubMed Database. Following MeSH terms were used in altering combinations: cagE, CagE, Helicopacter Pylori, Aggregatibacter actinomycetemcomitans, periodontitis, attachment loss, JP2, serotype. Articles were also supplied from our tutor Jan Oscarsson.

Statistical analysis
The statistical data are based on qualitative data. To determine the statistical significance of cagE prevalence in sick and healthy individuals and in healthy individuals with or without progression involves analyzing two or more major independent groups. The test that was carried out was thus Pearson chi-square test (McHugh, 2013). To determine the statistical significance of cagE pervalence in sick individuals without progression we analyzed two or more minor independent groups. The test that was carried out was thus Fisher’s exact test (Connelly, 2016). The tests were made using the software Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA).

Ethical consideration
The bacterial strains used in this study were collected during a cohort study of children and adolescents in Ghana (Åberg et al., 2012). The samples were gathered under ethical approval and the patients informed consent. Patient data was linked to bacterial samples that were used but the data could not be linked to the identity of the original patients. This project is not expected to lead to any direct benefit for the individuals the bacterial samples were collected
from, however this research may eventually lead to improved diagnostic methods to benefit patients in the future.

RESULTS

Presence of \textit{cagE} in the \textit{A. actinomycetemcomitans} strain collection

Of the 249 \textit{A. actinomycetemcomitans} isolates it was possible to test all but 5 by PCR and electrophoresis. After electrophoresis of the remaining 244 isolates a strong PCR amplicon band of approximately 1000 bp, consistent with the positive control could be detected in 23 of the isolates. These were classified as positive for the \textit{cagE} gene. For a number of isolates a weaker DNA band of approximately the same size could sometimes be detected. As we were unable to obtain a clear result for those strains despite several repeated experiments this PCR result was not considered as positive for the \textit{cagE} gene. A typical electrophoresis result is shown in Fig.1. The results of the PCR screening were compiled and cross run with the previously collected Ghana cohort data on periodontal disease at baseline, follow-up and progression (Åberg \textit{et al.}, 2012) (Table 1).

Presence of \textit{cagE} in relation attachment loss at baseline

At baseline (Table 1a), 174 of the 244 isolates were not associated with periodontal disease while for 70 of the isolates an attachment loss exceeding 3 mm had been measured. Of the health-associated isolates the \textit{cagE} gene was found in 16 (9 \%). Of isolates associated with registered attachment loss was \textit{cagE} present in 7 (10 \%). Statistically no significant difference was found with regard to the prevalence of the \textit{cagE} gene in isolates sampled from periodontally healthy individuals compared to diseased patients (p-value = 0.846 at a significance level of 0.05 (Pearson Chi square test)).

Presence of \textit{cagE} in relation to attachment loss at follow up

At follow-up (Table 1b) the number of \textit{A. actinomycetemcomitans} isolates associated with attachment loss was 101 (56 \%) of the total 180, while health-associated isolates were 79 (44 \%). The \textit{cagE} gene was found in 4 cases (5 \%) of isolates from healthy individuals and in 13 cases (13 \%) where attachment loss was present. Again, there was no statistically significant difference between periodontally healthy and diseased individuals in terms of prevalence of the \textit{cagE} gene (p-value 0.075 at a significance level of 0.05 (Pearson Chisquare test)).
Presence of cagE in relation to attachment loss progression in all cohort subjects

Comparing the attachment loss between baseline and follow-up (Table 1c), 78 (43 %) of the A. actinomycetemcomitans isolates were associated with a progression of attachment loss exceeding 3 mm compared to baseline, while 102 (57 %) of the isolates could not be linked to such progression. In only 5 (5 %) of the 102 health-associated isolates the cagE gene was present, while the gene was to be found in 12 (15 %) of the 78 isolates associated with disease progression. Statistically, there was a significant difference for the presence of the cagE gene in isolates associated with progression of attachment loss compared to isolates not associated (p-value 0.017 at a significance level of 0.05 (Pearson Chisquare test)).

Presence of cagE in relation to attachment loss progression in individuals healthy at baseline

Comparing the attachment loss between baseline and follow-up in samples with no attachment loss at baseline (Table 1d), 41 (32 %) of the A. actinomycetemcomitans isolates were associated with a progression of attachment loss exceeding 3 mm compared to baseline, while 87 (68 %) of the isolates could not be linked to such progression. In 5 (6 %) of the 87 isolates with no attachment loss the cagE gene was present, while the gene was to be found in 7 (17 %) of the 41 isolates associated with disease progression. Statistically, there was a significant difference for the presence of the cagE gene in isolates associated with progression of attachment loss compared to isolates not associated (p-value 0.04 at a significance level of 0.05 (Pearson Chisquare test)).

Presence of cagE in relation to attachment loss progression in individuals diseased at baseline

Comparing the progression of attachment loss between baseline and follow-up in samples with clinically registered attachment loss at baseline (Table 1e), 37 (71 %) of the A. actinomycetemcomitans isolates were associated with a progression of attachment loss exceeding 3 mm compared to baseline, while 15 (29 %) of the isolates could not be linked to such progression. The gene was to be found in 5 (14 %) of the 32 isolates associated with disease progression while 0 (0 %) of the 37 isolates with no progression carried the gene. Statistically, there was no significant difference for the presence of the cagE gene in isolates, diseased at baseline, associated with progression of attachment loss compared to isolates not associated (p-value 0.305 at a significance level of 0.05 (Fisher’s exact test)).
**Presence of cagE in relation to serotype**

When analyzing in which *A. actinomycetemcomitans* serotypes the cagE gene was present, a clear division of the isolates could be seen, where a majority of the 18 cagE-positive isolates (78%) belonged to serotype b (Table 2). The remaining cagE-positive isolates were distributed in serotype a (2 of them) and serotype c (3 of them). For serotype d, e and f the cagE gene was not found in any of the isolates.

**Presence of cagE in relation to leukotoxicity levels**

The leukotoxic activity of the 249 strains was earlier determined (Åberg et al., 2014b) Based on the isolates’ leukotoxicity, divided into low, medium and high, respectively (Table 3) much of the cagE- positive isolates were highly leukotoxic. 13 (59%) of the 22 cagE-positive isolates were high, 3 (14 %) medium, and 6 (27 %) had low leukotoxicity. In contrast, the distribution of the cagE- negative isolates, 10 (5 %) were high, 21 (10 %) medium, and 177 (85 %) had low leukotoxicity. One of the 23 cagE positive isolates was not earlier assayed for leukoxocicity. Thus, high leukotoxicity appears to be a property of cagE-positive rather than cagE-negative *A. actinomycetemcomitans* strains.

In summary, the cagE gene appeared to be prevalent mainly in *A. actinomycetemcomitans* serotype b strains, which exhibit high leukotoxicity. Consistent with this observation, a significant correlation between presence of the cagE gene in the *A. actinomycetemcomitans* strain carried by individuals of the Ghana cohort and a progression of attachment loss could be concluded. Thus, the cagE gene could function as a bacterial marker for risk for attachment loss.

**DISCUSSION**

The goal of this study was to investigate the prevalence of the cagE gene in a collection of isolates of *A. actinomycetemcomitans* collected from young adolescents in Ghana, and its possible use as a virulence marker. This gene was selected as studies have shown that cagE is conserved in the genome of certain *A. actinomycetemcomitans* strains and exhibited cytoxic and apoptotic activity (Teng and Hu, 2003; Teng and Zhang, 2005). Also, another study (Day et al., 2000) demonstrated a correlation between the cagE gene in *H. pylori* and the expression of virulence factors which aggravate the inflammation process at ulcerative
duodenitis. The function and the presence of the cagE gene in A. actinomycetemcomitans strains and serotypes had been little investigated and no studies were found in which the cagE gene has been researched in relation to clinical data and the presence of periodontal disease.

With the hypothesis that the cagE gene could serve as a marker of risk for periodontal disease the bacterial samples were analyzed via PCR. The result of the PCR was then compared with the previously collected clinical data of the adolescents in Ghana in regard of attachment loss and the progression of attachment loss at baseline and at follow-up two years later. A statistical analysis was then made to determine whether there was any statistically significant relationship.

The results of the analysis showed no relationship between the possession of the cagE gene and periodontal disease in the form of existing attachment loss before or after the follow-up. In contrast, a statistically significant association was seen between possession of the cagE gene and progression of attachment loss after 2 years in the subjects of the Ghana cohort. Dividing the subjects in two groups, there was a significant association between cagE and attachment loss in the individuals not exhibiting attachment loss at baseline, whereas such association could not be seen in individuals with a registered attachment loss at baseline.

We therefore concluded from this study that the cagE gene could function as a bacterial marker for initiation of the disease, i.e. a similar role as suggested for H. pylori cagE (Ramis et al., 2013). The statistically significant difference between cagE- positive and cagE- negative isolates regarding progression in patients healthy at baseline involves the potential use of the cagE gene as a marker to predict development of disease in a person without registered attachment loss but being colonized by a cagE-positive A. actinomycetemcomitans strain. Thus it can be speculated that CagE might play a role in the initiation of the periodontal disease, acting as a virulence factor to promote A. actinomycetemcomitans colonization whereas it is less important at later stages of the disease. It remains to be elucidated which possible role CagE may have in this disease.

With regards to serotype it was concluded that cagE gene positive isolates were all distributed in serotypes a, b and c with the largest majority in the serotype b, whereas none of the cagE-positive strains were classified as serotype d, e or f. This is consistent with the large genetic variability of this organism (Kaplan et al., 2002). An interesting observation was that even the
A. actinomycetemcomitans isolates which, after PCR analysis, showed a faint amplicon band of approximately the same size as the cagE-positive also were found distributed within serotypes a, b, and c. These, however, showed a different distribution where most of them were sorted to serotype a and c, rather than b. The explanation for this result is not known, and has been unprocessed further in this study. However, it may reflect binding of the PCR primers to other regions of the chromosome, unrelated to cagE. The faint bands were in this work therefore not used as positive indication for cagE.

Upon examining the earlier collected data on the expression of leukotoxin by the A. actinomycetemcomitans isolates analyzed in this study, a correlation could be seen between high leukotoxicity and presence of the cagE gene. Indeed, a clear majority of the cagE-positive, in contrast to the cagE-negative isolates were highly leukotoxic. This is in agreement with that several of the cagE-positive strains are of the highly leukotoxin genotype JP2 (Åberg et al., 2014a; Åberg et al., 2014b). Although the genetic background explaining why non-JP2 cagE-positive isolates were highly leukotoxic is still not known, it can be hypothesized that this feature is an important virulence factor in the cagE-positive strains, contributing to their association with attachment loss progression. Since cagE appears to be associated with elevated levels of leukotoxin, the gene may be helpful in finding such strains and could possibly serve as a genetic marker for enhanced leukotoxic ability and thus also for an increased risk for progression of attachment loss.

Conclusion

The aim of this work was to investigate a possible association between the possession of the cagE gene in strains of A. actinomycetemcomitans and presence of and/or progression of periodontal disease in the carriers. Based on our results we concluded that cagE could potentially serve as a bacterial marker for development of disease and early progression of attachment loss associated with periodontal disease. Thus, a PCR-based diagnostic test for the cagE gene might be useful to early diagnose patients at risk for aggressive progression of periodontal disease.
ACKNOWLEDGMENTS

We would like to thank our tutor Jan Oscarsson for support and guidance. We would also like to thank Chrissie Roth for aiding us in our laboratory work and Anders Johansson together with Rolf Claesson for their help and advice.
REFERENCES


Tables

Table 1. Illustrates the distribution of *cagE*-positive and *cagE*-negative *A. actinomycetemcomitans* strains carried by periodontally healthy and diseased patients at baseline and follow-up. The table further illustrates the distribution with regard to progression in, all samples combined and also in healthy and diseased samples separated.

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>cagE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) <em>cagE</em> in relation to periodontal status at baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy Baseline</td>
<td>16</td>
<td>158</td>
<td>244</td>
</tr>
<tr>
<td>Diseased Baseline</td>
<td>7</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>b) <em>cagE</em> in relation to periodontal status at follow-up</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy Follow-up</td>
<td>4</td>
<td>75</td>
<td>180</td>
</tr>
<tr>
<td>Diseased Follow-up</td>
<td>13</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>c) <em>cagE</em> in relation to AL progression (all subjects)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progression</td>
<td>12</td>
<td>66</td>
<td>180</td>
</tr>
<tr>
<td>No Progression</td>
<td>5</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>d) <em>cagE</em> in relation to AL progression (healthy at baseline)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Healthy Progression</td>
<td>7</td>
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<td>128</td>
</tr>
<tr>
<td>Healthy No Progression</td>
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<td>82</td>
<td></td>
</tr>
<tr>
<td>e) <em>cagE</em> in relation to AL progression (diseased at baseline)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Diseased Progression</td>
<td>5</td>
<td>32</td>
<td>52</td>
</tr>
<tr>
<td>Diseased No Progression</td>
<td>0</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Illustrates the distribution of \textit{cagE} between the different serotypes of \textit{A. actinomycetemcomitans} isolated from the Ghana cohort. The faint bands have in this work therefore not been used as positive indication for \textit{cagE}.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{cagE} No</td>
<td>53</td>
<td>26</td>
<td>90</td>
<td>12</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>\textit{cagE} Yes</td>
<td>2</td>
<td>18</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{cagE} Faint</td>
<td>4</td>
<td>1</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3. Illustrates the distribution of *cagE* between the different leukotoxicity phenotypes of *A. actinomycetemcomitans* isolates from the Ghana cohort.

<table>
<thead>
<tr>
<th>Leukotoxicity</th>
<th>cagE</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td></td>
<td>177</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>3</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Faint</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Figures

Figure 1. A representative image of PCR analysis of *cagE* using the forward and reverse primer. The red arrow is pointing out a typical band indicating the *cagE* gene in a test sample. The orange arrow is marking a positive control: DNA from a JP2-clone named HK 1651 (expected size is 1020 bp). As negative control (blue arrow) H₂O was used. The faint amplicon band sometimes detected, and in this study not considered as a positive indicator for *cagE* is shown by the yellow arrow. The letter M (Molecular weight size marker) is in the figure positioned above the lane in the gel where this marker was loaded.