Microfluidic analysis of multiplex PCR products for the genotyping of Helicobacter pylori

Application

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Abstract

Gastric infection with Helicobacter pylori (H. pylori) is the major cause of chronic active gastritis and is associated with the pathogenesis of peptic ulcer and gastric carcinoma. Recent studies using a multiplex PCR (mPCR) based approach distinguished different allelic variants that are associated with different stages of H. pylori virulence. H. pylori genotyping on gastric tissues, routinely processed in diagnostic pathology, revealed that certain combinations of virulence subtypes of different H. pylori sub-strains are associated with gastric carcinoma and MALT lymphoma. The use of formalin fixed samples as the source for template DNA for mPCR restricts the size of the PCR fragments to 300 bp, due to poor DNA quality. For the analysis of multiple bands of a mPCR experiment, the data acquisition from slab gels is often inconvenient in terms of sizing, resolution, and data accuracy. To improve accuracy and reproducibility of the measurements, the Agilent 2100 bioanalyzer using Lab-on-a-chip technology was tested to replace slab gels as the tool for the electrophoretic separation of the mPCR products. First analyses revealed an improved fragment resolution, sizing accuracy and reproducibility. A mPCR reaction covering five different alleles that are involved in H. pylori pathogenesis was analyzed and all fragments were separated and quantified. The analysis of a mixture of all mPCR reactions including all involved alleles showed the separation of seven different PCR fragments in the range of 102 to 301 bp, thereby expanding the spectrum of prognostic or therapeutically relevant information used e.g. in H. pylori diagnostic.

Introduction

The human pathogen Helicobacter pylori (H. pylori) is associated with the development of a variety of gastro-duodenal diseases, such as chronic active gastritis, peptic ulcer disease, gastric cancer, and gastric mucosa associated lymphoid tissue (MALT) lymphoma[1] It is estimated that about half of the world's population is infected with H. pylori. But despite the high prevalence of infections only a minority of infected individuals will develop gastric carcinoma or MALT lymphoma. Therefore, it is essential to identify factors that might determine the possible sequelae of the H. pylori infection, such as host and environmental factors and bacterial virulence genes.

The specific H. pylori virulence genes found to be associated with possible disease outcome are the various alleles of the vacA gene, the presence of the cagA gene as a marker for a pathogenicity island, and the allelic variants of the iceA gene. The vacA gene is present in all H. pylori strains and encodes for the vacuolating cytotoxin, VacA which causes vacuolar degeneration of gastric epithelial cells. The vacA gene, although present in all strains, differs in its allelic variants in the signal region and the mid-region of the gene. The sequence variations in the signal region are distinguished into

vacA s1 and vacA s2 subtypes, and the mid-region can be distinguished into m1 or m2 subtypes[2]. The allelic variants of the vacA gene that were detected by nested PCR were vacAs1/m1, vacAs1/m2 and vacAs2/m2. These vacA gene variants are associated with different cytotoxin activities. The cagA gene is a marker for the presence of the cag pathogenicity island, which is a 40 kb locus containing 31 genes associated with the induction of interleukin 8 secretion by gastric epithelial cells[3]. The allelic variants of the iceA gene are distinguished into iceA1 or iceA2 and only one of which being found in all H. pylori strains. The iceA gene seems to be induced by contact with epithelium and associated with increased mucosal interleukin 8 concentrations[4].

In our studies[5], we have found that certain H. pylori subtype combinations of the vacA, cagA and iceA genes possess a differentiating and predictive value for the development of gastric adenocarcinoma and MALT lymphoma. We have established a multiplex PCR analysis to detect and distinguish the different H. pylori genes and their allelic variants from DNA extracted from paraffin wax embedded tissues. The use of template DNA derived from formalin fixed and paraffin wax embedded tissue for the mPCR restricts the size of the PCR fragments to a maximum of about 300 bp due to fragmentation and poor DNA quality from such tissue. The primer sets used in the mPCR for the detection of the different H. pylori virulence genes generated amplification products with a maximum length of 301 bp. The combined use of primer sets in the mPCR was possible because each primer set required an annealing temperature of 58 °C and all PCR products were distinguishable by size on slab gel electrophoresis. In order to obtain more reproducible and standardized results, a microfluidic based analytical platform, the Agilent 2100 bioanalyzer, using lab-on-a-chip technology[6] was tested to replace slab gels as the tool for the electrophoretic separation of the mPCR products.

Material and methods

Tissues

The paraffin embedded and formalin fixed gastric tissues from chronic gastritis, gastric adenocarcinoma and gastric MALT lymphoma specimen were recruited from the archives of the Institutes for Pathology of the University of Mainz (Germany) and Cologne (Germany). Three 5-7 µm-sections of each specimen were de-waxed in xylene and the nucleic acids were extracted by phenol/chloroform followed by precipitation in 300 mM sodium acetate and 50 % isopropanol.

PCR

For H. pylori genotyping, a sequence specific, nested PCR approach was performed as described earlier by Koehler et al. [5]. Briefly, genomic sub-fragments of the virulence factors vacA, cagA, and iceA were amplified by the combined use of allele-specific primer sets (vacA s1/s2, vacA m1/m2, cagA, and iceA 1/2) in two PCR amplification steps, each starting with a preincubation at 95°C for 5 minutes, then followed by 35 cycles of 1 minute denaturation at 95 °C, 1 minute annealing at 58 °C and 1 minute extension at 72 °C. PCR yielded products with sizes varying from 102 bp to 301 bp.

Analysis of PCR-products

PCR products were analyzed using standard agarose gel electrophoresis and Lab-on-a-chip technology on the Agilent 2100 bioanalyzer using the Agilent DNA 1000 LabChip® kit according to the manufacturer's instructions.

Results and discussion

In a previous study [5] we have established a nested multiplex PCR (mPCR) assay in order to identify the different virulence factors of Helicobacter pylori, vacA, cagA and iceA genotypes in routinely processed gastric biopsies which are formalin fixed and paraffin embedded. The nested mPCR assays with a limited number of primer sets produced reliable, distinct amplification products of all specimens. The combined PCR assays for the simultaneous detection of the vacA mid-regions m1 and m2, as well as the combined detection of iceA1 and iceA2 were routinely performed. The semi-nested PCR for the detection of the vacA signal regions s1 and s2 was usually combined with the nested PCR for the cagA gene. The established mPCR assay permits the simultaneous analysis of all seven different diagnostically relevant alleles. However, tested on a broad spectrum of diagnostic samples and analysed on a standard agarose gel, the assay resulted in PCR fragment patterns that were difficult to interpret in some cases.

To increase analytical precision of these studies the Agilent 2100 bioanalyzer, using Lab-on-a-chip technology was tested to replace slab gels as the tool for the electrophoretic separation and analysis of the mPCR products. First, we compared the analysis of the routinely performed combined PCRs of vacAs1/s2/cagA, of vacAm1/m2 (data not shown) and of iceA1/2 (figure 1) on the Agilent 2100 bioanalyzer with slab gel results. The microfluidic analysis of the PCR amplicons of the H. pylori virulence genes showed and confirmed the results of our samples as imaged by slab gel electrophoresis. Especially the improved fragment resolution by the Agilent 2100 bioanalyzer (figure 1B) compared with the slab gel analysis (figure 1A) and the representation as an electropherogram (figure 1C) were helpful in differentiating DNA fragments, which differ in size by only 29 bp as with iceA1 and iceA2 (figure 1).

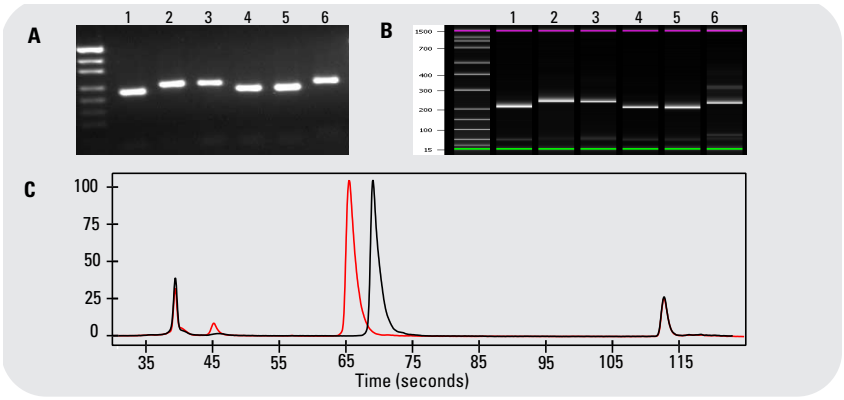

Figure 1: Comparison of the iceA1/A2 PCR analysis on the Agilent 2100 bioanalyzer with slab gel results. A) Slab gel analysis of the iceA1 and iceA2 allele PCR. The difference of the detected alleles is 29 bp. A slight drift in the position of the fragments on the gel is detectable complicating the discrimination of the two alleles. B) Gel-like image of the ice mPCR analyzed on the Agilent 2100 bioanalyzer. C) Overlay of two electropherograms representing the analysis results of the iceA1 and the iceA2 alleles on the bioanalyzer. Both alleles are clearly distinguishable.

Next, the combined analysis of the five different alleles (vacA s1 or s2, cagA, vacA m1 or m2) was investigated (figure 2). Whereas the detection of the 5 alleles of H. pylori genotypes was sometimes difficult when imaging was performed with slab gel electrophoresis the imaging of microfluidic data showed improved analysis of distinct amplification products. The gel-like image shows the separation of vacAs2/vacAm2 in lane 1 and 4 and vacAs1/cagA/vacAm1 in lane 2 and 3 (figure 2A). The analysis resulted in calculated sizes of 98 and 101 bp for the vacAm2 amplicon and in calculated sizes of 151 and 148 bp for the vacAs2 amplicon (expected sizes: vacAm2: 102 bp and vacAs2: 150 bp, respectively). In addition to

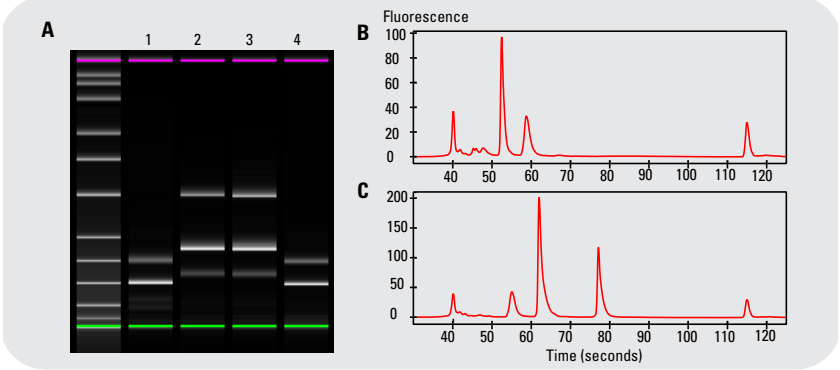

Figure 2: Microfluidic analysis the 5-plex mPCR. Five different alleles: vacA s1 or s2, cagA, vacA m1 or m2 were amplified and analyzed on the Agilent 2100 bioanalyzer. The targets were detected with high fragment resolution. A) Gel-like image (lane 1 + 4: vacAs2/vacAm2; lane 2 + 3: vacAs1/cagA/vacAm1). B) Electropherogram of lane 1 of gel like image showing the separation of the amplified vacAs2/vacAm2 alleles. C) Electropherogram of lane 2 of the gel-like image showing the separation of the amplified vacAs1/cagA/vacAm1 alleles.

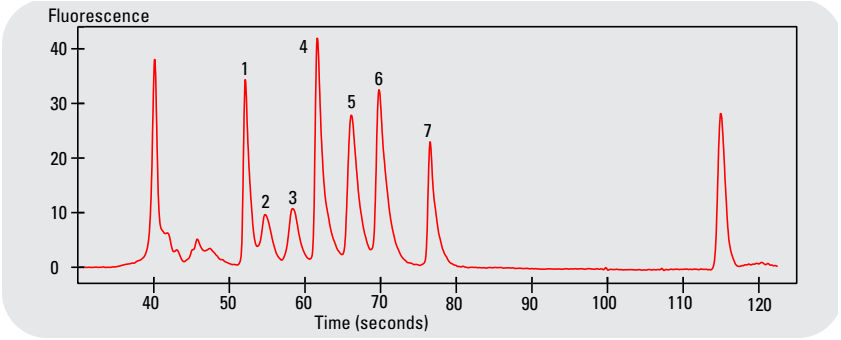

Figure 3: Combined analysis of four different mPCR reactions. The combined mPCR contains seven fragments within a range of 102-301 bp. All fragments could be separated and quantified on the Agilent 2100 bioanalyzer.

the gel like image, the result of lane 1 and 2 of figure 2A are displayed as electropherograms (figures 2B and 2C).

Finally, combined microfluidic analysis of all 7 alleles of H. pylori amplified in 4 different mPCRs was possible on the chip after mixing all mPCR reactions prior to analysis (combination of: iceA1/A2, vacA s1/s2, cagA, vacA m1/m2). This is difficult with slab gel electrophoresis where differences in the amount of PCR products cause difficulties in data interpretation. All seven different mPCR products within a range of 102-301 bp could be separated and quantified (figure 3). These results reveal that Lab-on-a-chip technology enables the separation of seven different targets restricted to a size range up to 300 bp in a combined mPCR reaction.

Conclusion

The results of this study clearly demonstrate the ability of the Agilent 2100 bioanalyzer to differentiate the allelic H. pylori variations, vacAs1/s2/cagA, of vacAm1/m2 and of iceA1/2, amplified by mPCR. The high resolution achieved by the microfluidic based Lab-on-the-Chip technology allows to analyze a broad and expanding panel of virulence and risk factors. This is of great importance since an extended spectrum of prognostic or therapeutically relevant information used in H. pylori diagnostics will now be accessible for routinely processed simultaneous diagnostics by means of mRCR assays.

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