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Citation for published version (APA):

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Chapter 3

Analysis of the capsular polysaccharide biosynthesis locus of *Porphyromonas gingivalis* and development of a K1-specific PCR-based serotyping assay

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Published partly in *Journal of Periodontal Research* 2008; 6: 698-705
Abstract

*Porphyromonas gingivalis* is a Gram negative obligate anaerobe that is strongly associated with severe periodontitis. Previous reports showed an association of *P. gingivalis* capsular polysaccharide with virulence. The K1 capsular polysaccharide was found to be more immunostimulatory than the other serotypes. Our objective was to explore the genetic background of the capsule biosynthesis (K-antigen) locus in a representative group of K1 serotype strains.

We used restriction fragment length polymorphism, polymerase chain reaction (PCR) and DNA sequencing to study the capsular polysaccharide locus in *P. gingivalis* K1 strains. For serotyping by double immunodiffusion and PCR we used 32 strains of *P. gingivalis*, including strains of all six known K serotypes.

All tested K1 strains showed high conservation of the capsular polysaccharide locus, although a DNA re-arrangement was found in two strains. Based on this information a K1-specific PCR-based serotyping assay was designed. The specificity and sensitivity of this test were confirmed using non-K1 *P. gingivalis* serotypes.

The new K1 serotyping assay presented here is much faster than double immunodiffusion and can detect K1 strains in a very selective and sensitive way. When using complex patient samples K1 strains are also detected. This method may therefore be clinically relevant in the detection of the virulent *P. gingivalis* K1 serotype.

Introduction

Periodontitis is an inflammatory disease of the tooth-supporting tissues that is caused by dental plaque bacteria. The disease is characterized by inflammation of the gums and loss of alveolar bone. It is a multifactorial disease in which host genetic traits and behavioral factors, such as smoking, are considered as risk factors. Periodontitis has been associated with cardiovascular diseases [1,2], preterm low birth weight [3,4] and pre-eclampsia [5,6]. Among the great number of dental plaque bacteria, the Gram negative, often encapsulated, strict anaerobe, *Porphyromonas gingivalis*, is strongly associated with the disease [7,8]. The pathogen displays a wide variety of virulence factors, including Arg-gingipains and Lys-gingipains, fimbriae, haemagglutinins, lipopolysaccharides and capsular polysaccharides [9].
Mouse model studies have shown that encapsulated *P. gingivalis* strains are more virulent than nonencapsulated strains [10–14]. Encapsulated strains cause spreading infections, whereas nonencapsulated strains tend to cause localized abscesses [13]. The capsular polysaccharide is believed to function as an immunogen [15,16] because mice, immunized with purified capsular polysaccharide, are protected from oral bone loss after a challenge with *P. gingivalis*.

To date, six capsular serotypes (K1–K6) have been described [17,18], which have been examined for prevalence in periodontitis patients and for virulence in a mouse model [11,19]. A seventh serotype (K7) has been suggested by R. E. Schifferle [personal communication]. Currently, little is known about the differences in capsular polysaccharide structure between serotypes. A recent study reported on murine peritoneal macrophages that induced more chemokine expression when challenged with *P. gingivalis* K1 serotype capsular polysaccharide (W50 and W83) than when challenged with capsular polysaccharide of other serotypes. In addition, it was shown that these chemokines also contributed to the migration of polymorphonuclear leukocytes [20]. This demonstrates that the K1 capsular polysaccharide plays an important role in host–pathogen interaction.

Previously, Farquharson and co-workers, and Schifferle and co-workers, showed that the sugar composition of K1 (W50) and K3 (A7A1-28) serotype capsular polysaccharide is totally different [21,22]. The K1 strains may therefore have a capsular structure that is more immunostimulatory than the other *P. gingivalis* serotypes.

Little is known about the genetic background of capsule biosynthesis in *P. gingivalis*, but publication of the genome sequence of the K1 strain, W83, has provided new insights and has revealed at least four potential capsular polysaccharide biosynthesis loci [23]. Further genetic information was obtained from a comparison of the encapsulated W83 K1 and the nonencapsulated ATCC33277 strains by whole-genome analysis. Strain ATCC33277 was found to lack a cluster of open reading frames (PG0106–PG0120) that is believed to be involved in capsular polysaccharide synthesis in W83. This finding is the first evidence to indicate that this set of open reading frames is a potential genomic capsular polysaccharide biosynthesis locus [24]. The experimental evidence came from a recent study in which Aduse-Opoku and co-workers [25] identified the capsular polysaccharide locus of *P. gingivalis* by making mutants that lacked several genes from the PG0106–PG0120 locus. These mutants lack a capsule and no longer showed reactivity with the K1 antiserum [25]. Restriction fragment length
polymorphism of the capsular polysaccharide locus suggested that there is strong variation between serotypes, but only little within serotypes. This potential variation in gene content between the serotypes in the capsular polysaccharide locus may be used to explain the differences in sugar contents of the serotypes.

The genetic locus for capsule biosynthesis has been described and the first functional studies of the genes have been performed [25,26]. Little variation in the capsular polysaccharide locus within a serotype was suggested. Considering the clinical relevance of accurate K1 serotyping, a more detailed knowledge of the capsular polysaccharide locus is warranted. Therefore, the aim of the current study was to examine the homogeneity in the capsular polysaccharide biosynthetic locus of a significant number of K1 serotype strains by restriction fragment length polymorphism typing and gene-specific polymerase chain reactions (PCRs). This detailed examination of the K1 capsular polysaccharide biosynthesis locus allowed us subsequently to develop a PCR-based serotyping method to identify the K1 P. gingivalis serotype.

Material and Methods

Bacterial physiology and maintenance

Strains that were used in this study are described in table 1. All P. gingivalis strains were isolated from patients with moderate to severe periodontitis. These strains were grown either on 5% horse blood agar plates (Oxoid no. 2; Oxoid, Basingstoke, UK) or brain–heart infusion broth, both supplemented with hemin (5 µg/mL) and menadione (1 µg/mL), in an anaerobic atmosphere of 80% N₂, 10% H₂ and 10% CO₂. Escherichia coli DH5α was used for maintenance of plasmids. E. coli DH5α was cultured in Luria–Bertani broth or on solid medium (Luria–Bertani broth with the addition of 1.5% agar). Ampicillin (Na⁺ salt; 100 µg/mL) was added to the growth media to select for pUC-derived plasmids.

Genomic DNA isolation from P. gingivalis

Genomic DNA from P. gingivalis strains was isolated from plate-grown bacteria using the DNeasy tissue kit (Qiagen Benelux BV Qiagen GmbH, Hilden, Germany). The DNA concentration of all samples after purification was between 20 and 60 ng/µL.
Restriction fragment length polymorphism

Genomic DNA from *P. gingivalis* K1 strains was isolated as described above. DNA was purified by an extra ethanol/NaCl precipitation to yield concentrated DNA with a 260/280 nm ratio of 1.9 or higher. Extensor high-fidelity PCR master mix (ABgene House, Surrey, UK) was used to amplify a 15.4 kb DNA fragment, corresponding to the capsular polysaccharide locus region *PG0106–PG0120*, using the primers *PG0106F3* and *PG0117R3* [25]. The PCR was performed in a Dyad Disciple thermocycler (MJ Research, Waltham, MA, USA) using the PCR program 92°C for 2 min followed by 10 cycles at 92, 55 and 68°C for 10 s, 30 s and 12 min, respectively, and then 20 cycles at 94, 55 and 68°C for 10 s, 30 s and 12 min (+10 s/cycle), respectively, followed by a final step at 68°C for 7 min. Amplicons were purified and concentrated by ethanol/NaCl precipitation and dissolved in TE (10 mM Tris, 1 mM EDTA) buffer (pH 8). One microgram of DNA was
restricted with 10 U of \textit{BglII}. (New England Biolabs Ltd., Herts, UK). The restriction patterns were then analyzed by electrophoresis on a 1% agarose TBE (100 mM Tris, 1 mM EDTA, 90 mM boric acid) gel in the presence of ethidium bromide.

\textbf{\textit{P. gingivalis} serotyping}

Serotyping of \textit{P. gingivalis} was based on the detection of the six described K-antigens \cite{17,18}. In short, serotype-specific, polyclonal antisera were obtained after immunization of rabbits with whole bacterial cells \cite{27} of the six \textit{P. gingivalis} type strains. Bacterial antigens for double immunodiffusion and immunoelectrophoresis tests were prepared as described previously \cite{17}.

\textbf{Gene-specific PCR reactions}

In addition to the restriction fragment length polymorphism analysis, we used gene-specific primers (table 2) for the 13 genes in the capsular polysaccharide locus to confirm the presence of the expected genes in the K1 test strains. A standard PCR setup was used, in which \textit{Taq} DNA polymerase (Fermentas GmbH, St Leon-Rot, Germany) was used to amplify genes from 100 ng of genomic DNA. The program started with an incubation at 95°C for 5 min and then 30 cycles of incubation at 95, 55 and 72°C for 30 s, 30 s and 2 min, respectively, and ended by one incubation step at 72°C for 5 min. The amplicons varied in length from 0.5 to 1.7 kb.

\textbf{DNA sequencing and analysis}

Two, 2.4-kb \textit{BglII} fragments of the PCR amplicons of K1 strains OMGS1577 and OMGS2104 were isolated from a 1% TAE (40 mM Tris-acetate, 10 mM EDTA) agarose gel using a gel extraction kit (Qiagen Benelux BV), purified by ethanol precipitation and ligated into a \textit{BamHI}-digested pUC19 cloning vector using T4 DNA ligase (Fermentas GmbH). The constructs were transformed into \textit{E. coli} DH5α using a Electro Cell Manipulator 600 (BTX Instrument Division, Holliston, MA, USA; 25 µF, 2.5 kV, 200 Ω). The plasmids were isolated using a QIAPrep spin miniprep kit (Qiagen Benelux BV). The inserts were completely sequenced using the primers MF, MR, PG0114FW Seq and PG0114R Seq. Sequencing was carried out with BigDye 3.1 terminator chemistry (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) and resolved using a 48-capillary ABI PRISM 3730 (Applied Biosystems) DNA sequencer. Analysis of the
sequence data was performed using the VECTORNTI software package (Invitrogen, Breda, the Netherlands).

**K1 serotype-specific PCR method**

Eight primer combinations (Sero0116F2 × Sero0117F3, Sero0116F3 × Sero0117R2, Sero0116F3 × Sero0117F3, Sero0117F1 × Sero0118R2, Sero0117F1 × Sero0118R4, Sero0117F2 × Sero0118R2, Sero0117F2 × Sero0118R3 and Sero0116F3 × Sero0118R3; table 2) were designed in the variable 3'-region of the *P. gingivalis* capsular polysaccharide locus at
PG0116, PG0117 and PG0118. The shortest expected fragment was about 0.3 kb and the longest was 2.0 kb. A standard PCR setup with a 72°C elongation step of 3 min was used, in which Taq DNA polymerase (Fermentas GmbH) was used for amplification from 100 ng of genomic DNA. The positive control used in this experiment was a 68 bp fragment of the 16S rDNA of P. gingivalis (primers PgF and PgR, table 2). The PCR products were analyzed by electrophoresis on a 1% agarose TBE gel in the presence of ethidium bromide.

Pre-treatment of patient samples for K1-specific PCR

DNA isolated routinely for periodontitis diagnostics was used for the K1 specific PCR directly, but RTF samples had to be pre-treated in the following way. After centrifugation of the samples for 1 minute at 20,000 x g the pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Then, after boiling in a microwave for 1 minute, the sample was used as a PCR template in the K1 specific PCR reaction.

PCR-based serotyping of K1 P. gingivalis strains

We designed a primer set – PG0117F2 with PG0118R3 (table 2) – on the basis of sequencing results which showed that the combination of PG0117 and PG0118 is only present in K1 strains (W50 and W83) and not in AJW5 (K2), HG1703 (K4), HG1690 (K5) and HG1691 (K6) [24; J. Aduse-Opoku, personal communication]. This set was used for all strains to test the sensitivity and specificity for K1 strains of P. gingivalis. A standard PCR setup was used, in which Taq DNA polymerase (Fermentas GmbH) was used to amplify fragments from 100 ng of genomic DNA. The program started with an incubation at 95°C for 5 min and then 30 cycles of incubation at 95, 55 and 72°C for 30, 30 and 90 s, respectively, and ended with a final incubation at 72°C for 5 min. The expected amplicon size was 629 bp. A P. gingivalis-specific PCR on 16S rDNA was included as a positive control using primers PgF and PgR. The expected size of this amplicon was 68 bp in all P. gingivalis strains [28]. The amplicons were analyzed by agarose gel electrophoresis in the presence of ethidium bromide.
Results

Homogeneity of the capsular polysaccharide locus in K1 strains

It was possible to amplify the K-antigen locus from all K1 strains tested in this study using the primers PG0106F3 and PG0117R3. Each strain yielded an amplicon of about 15.4 kb (data not shown). After BglII restriction of the amplified DNA fragments, the restriction fragment length polymorphism patterns of all K1 strains were similar to that of the sequenced W83 strain (figure 1). Strains OMGS1577 (Japanese periodontitis isolate) and OMGS2104 (Chinese periodontitis isolate) showed a slightly different pattern in that both strains seemed to have one shifted band. Because both strains showed reactivity with K1 antiserum (figure 2), these strains were expected to contain the genes found in the capsular polysaccharide locus of W83.

Figure 1. Restriction fragment length polymorphism analysis of the 15.4 kb capsular polysaccharide loci of ten K1 strains. The figure shows a 1% agarose gel of BglII-digested amplicons of strains W83 (lane 1), W50 (lane 2), HG1631 (lane 3), HG1633 (lane 4), HG1634 (lane 5), HG1636 (lane 6), OMGS1577 (lane 7), OMGS1578 (lane 8), OMGS1746 (lane 9) and OMGS2104 (lane 10). M denotes the lane containing the GeneRuler™ DNA Ladder Mix (Fermentas GmbH, St Leon-Rot, Germany).
Presence of capsular polysaccharide locus genes in K1 serotype strains

We performed gene-specific PCRs on each of the 13 potentially functional K-antigen biosynthesis locus genes to study if all genes of the sequenced W83 strain (figure 3) are present in all K1 strains. Almost all genes could be detected by the gene-specific PCR method (table 3). Only in the case of strains OMGS1577 and OMGS2104 was additional evidence needed to examine whether all expected capsular polysaccharide biosynthesis genes were present. The small restriction fragment length polymorphism difference detected (figure. 1) was caused by the presence of a 2.4 kb fragment instead of a 1.7 kb fragment found in the typical K1 strains (i.e. strains W83, W50, HG1631, HG1633, HG1634, HG1636, OMGS1578 and OMGS1746 that display matching restriction fragment length polymorphism patterns). The organization of the capsular polysaccharide locus of strains OMGS1577 and OMGS2104 was elucidated by extra gene-spanning PCR reactions and after cloning and sequencing of the shifted 2.4 kb restriction fragment length polymorphism fragment. The analysis revealed that the fragment consisted of part (746 bp) of the PG0113 gene, the complete PG0114 gene, an unknown 122 bp fragment and a 570 bp fragment with high homology (> 95%) to the capsular polysaccharide biosynthesis locus of strain FDC381 [25] (figure. 4).
The combination of primer Sero0117F2 with Sero0118R3 was chosen from the eight primer sets because that primer combination yielded the best amplification of fragments in a small pilot experiment with K1 strains (data not shown). This PCR reaction yielded the expected 630 bp product with all 11 tested K1 serotype strains but not with any of the other *P. gingivalis* strains tested (figure. 5).

**K1 serotype-specific PCR**

From 175 samples tested by the K1 specific PCR 5 samples were positive, which is 2.9% of the *P. gingivalis* positive samples. In parallel all samples tested positive were cultured on blood agar plates and *P. gingivalis* strains were then serotyped using double immuno-diffusion to confirm the K1 serotype identity. From all five samples a K1 strain could be isolated.
The aim of this study was to investigate in more detail the capsular polysaccharide biosynthesis locus in K1 \textit{P. gingivalis} strains. Our interest was to study the homogeneity of the previously described capsular polysaccharide biosynthesis locus in strains of the K1 serotype of \textit{P. gingivalis} and to explore the possibility of developing a K1-specific PCR method. Significant heterogeneity between different \textit{P. gingivalis} serotypes was found in a previous study using restriction fragment length polymorphism [25]. The data suggested, however, that the K1 serotype locus may be relatively homogenous. To establish the homogeneity of the K1-capsular polysaccharide locus we used restriction fragment length polymorphism to type a significant number of K1 strains. We found that all strains tested had an identical restriction fragment length polymorphism.
pattern, except for the strains OMGS1577 and OMGS2104. The small difference that was observed in these strains was caused by the presence of a 2.4 kb fragment instead of the 1.7 kb fragment typically found in K1 strains. This fragment was further analyzed by sequencing. The analysis revealed that the fragment consisted of part of the \textit{PG0113} gene, the \textit{PG0114} gene and part of the \textit{PG1205} gene. This latter open reading frame was identical to the \textit{PG1205} gene that was found previously in the nonencapsulated strain, FDC381, and the protein is homologous (59% identity, 77% similarity) to DNA-binding proteins of the histone family. It was suggested that this gene might disrupt the transcription of the 3' end of the capsular polysaccharide biosynthesis locus, thereby having a negative effect on capsular polysaccharide expression [25]. However, when we combined the results from the restriction fragment length polymorphism and sequencing, it was found that in strains OMGS1577 and OMGS2104, \textit{PG1205} is located between genes \textit{PG0114} and \textit{PG0115}. So, our data indicate that in these strains the locus arrangement is the same as in strain FDC381, which does not support the suggestion that \textit{PG1205} interferes with downstream transcription, as both strains display a K1 serotype. Besides insertion of an extra gene, a restriction site was lost in gene \textit{PG0117} in these two strains. As the inserted \textit{PG1205} carries a \textit{BglII} restriction site, the restriction
fragment length polymorphism pattern, which actually changed significantly, appears similar to the typical K1 strain pattern.

To study the capsular polysaccharide K1 locus in greater detail, we performed gene-specific PCR reactions on ten K1 strains of *P. gingivalis*. These PCR reactions supported the restriction fragment length polymorphism results and endorsed the previous results in finding only little variation within the K1 serotype. All genes that were found in the capsular polysaccharide biosynthesis of the sequenced K1 strain, W83, were also found in the nine other strains.

Having recognized gene structure homogeneity within the K1 strains we continued with the development of a PCR-based serotyping method for K1.
Based on differences that can be found in the 3' end of the capsular polysaccharide biosynthesis locus [25] in different serotypes, we started with eight primer sets in genes PG0116, PG0117 and PG0118. The selected method is specific for the genes PG0117 and PG0118 positioned next to each other in the same orientation. We have shown that the sensitivity of this PCR, using primers Sero0117F2 with Sero0118R3, is optimal as our PCR detected all K1 strains. We analyzed the specificity of our method by PCR amplification reactions on the DNA of 32 P. gingivalis strains. The primers are optimally specific, as none of the non-K1 strains showed any reactivity. Interestingly, the nontypeable strain, HG1695, was included in this experiment. Our K1 PCR method did not detect this strain as a K1 strain, although in previous studies it was shown to have a restriction fragment length polymorphism pattern identical to the typical K1 strains [25]. This finding, of a similar capsular polysaccharide locus organization to the K1 strains but a difference in PG0117–PG0118, might indicate that either PG0117 or PG0118 genes, or both, are specifically important in the biosynthesis of the K1 antigen. PG0117, a flippase involved in polysaccharide biosynthesis, was absent in strains HG1703 (K3) and FDC381 (K−) [25], and thereby potentially K1 specific, but a mutation in this gene did not alter the serotype. PG0118, coding for a glycosyl transferase, is absent in HG1703 and has less than 65% identity with PG0118 (W83) in strain FDC381. These observations, combined with the specificity of the PCR based on PG0118 presented here, suggest that this gene is essential in defining K1 serospecificity. It was demonstrated in vitro that the K1 serotype capsular polysaccharide has a more challenging effect on macrophages and polymorphonuclear neutrophils than other serotypes [20] and hence plays an important role in host–pathogen interactions. The deduction of PG0118 as K1-essential, and the availability of an uncomplicated K1-specific serotyping assay, may be of clinical relevance. Screening of patient samples for the presence of K1- P. gingivalis by the newly developed PCR method yielded a prevalence of 2.9%. This is comparable with the 3.7% found in the same type of population by double immunodiffusion using a K1 antiserum [19]. Using this quick serotyping method in combination with analyses of severity and progression of the disease, may lead to more insights of the role of K1 serotype P. gingivalis strains in periodontitis.
Acknowledgements

We are grateful to J. Aduse-Opoku and M. A. Curtis for helpful discussions and critical review of the manuscript. We thank W. de Wit for analytical support.

References


