Periodontopathogens, Porphyromonas Gingivalis and Aggregatibacter Actinomycetemcomitans in placenta: a functional molecular diagnosis

Periodonopatógenos, Porphyromonas Gingivalis e Aggregatibacter Actinomycetemcomitans em placenta: um diagnóstico molecular funcional

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Resumo

A prematuridade está associada à morbidade e mortalidade neonatal. Suas principais causas clássicas, ainda não esclarecem todos os casos. As infecções verticais translocadas foram cogitadas. O diagnóstico molecular funcional de patógenos periodontais na placenta ainda não foi proposto. Pretende-se detectar a presença de microrganismos e a expressão de mRNAs específicos para RNA polimerase (RNApol) de Porphyromonas gingivalis e da leucotoxina de Aggregatibacter actinomycetemcomitans no tecido placentário de mulheres com doença periodontal e parto prematuro. O estudo prospectivo avaliou mulheres que apresentaram manifestações de doença periodontal e parto prematuro (≤ 36 semanas, n = 19) em um hospital-escola, na região centro-oeste do Brasil, em comparação com controles saudáveis (n = 10). Amostras de placenta foram obtidas

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logo após o parto. Foram desenvolvidos oligonucleotídios específicos para RT-qPCR. O método CT comparativo foi utilizado para análise de expressão de genes alvos, normalizados com RNA de S26 endógeno. Os resultados mostraram que a expressão de mRNA de leucotoxina de Aggregatibacter actinomyctemcomitans foi detectada em 31% amostras de placenta, enquanto a expressão de mRNA da RNApol de Porphyromonas gingivalis foi encontrada em 75% amostras. Nenhum desses genes foi detectado em placentas normais. Esses dados são de grande importância clínica, uma vez que a presença de periodontopatógenos na placenta e sua expressão funcional evidenciam que o microrganismo, que é de origem oral, teve a capacidade de translocação, sugerindo aqui, uma evidência da relação entre doença periodontal de a mãe e a prematuridade do recém-nascido.

Palavras-chave: Placenta; doença periodontal; Porphyromonas gingivalis, Aggregatibacter actinomyctemcomitans; qPCR; transcrição reversa, expressão gênica.

Abstract
Prematurity is associated with neonatal morbidity and mortality. Its main classic causes, not yet clarify all cases. Translocated vertical infections have been cogitated. Functional molecular diagnosis of periodontal pathogens in placenta has not yet been proposed. We aimed to detect the presence of microorganisms and the expression of specific mRNAs as for RNA polymerase (RNApol) of Porphyromonas gingivalis and leukotoxin from Aggregatibacter actinomyctemcomitans in the placental tissue of women with periodontal disease and preterm birth. The prospective study evaluated women, who had manifestations of periodontal disease and preterm labor (≤ 36 weeks, n=19) in a school hospital in the central-western region of Brazil compared to healthy controls (n=10). Placental samples were taken shortly after delivery. Specific oligonucleotides were developed for RT-qPCR. Comparative CT method was used for expression analysis of target genes normalized with endogenous S26 RNA. The results showed that Aggregatibacter actinomyctemcomitans leukotoxin mRNA expression was detected in 31% of placenta tested samples, while RNApol mRNA expression of P. gingivalis was found in 75%. None of these genes were detected in normal placentas. This data is of major clinical importance, since the presence of periodontopathogens in the placenta and its functional expression, evidences that the microorganism, which is of oral origin, had the capacity of translocation, suggesting here, an evidence of the relation between periodontal disease of the mother, and the prematurity of the newborn.

Key words: Placenta; periodontal disease; Porphyromonas gingivalis, Aggregatibacter actinomyctemcomitans; qPCR; reverse transcription, gene expression.

Introduction
Epidemiological data indicate that thirty to fifty percent of cases of prematurity are triggered by distance infection (Bittar and Zugai, 1993; Passini et al., 2007). Current studies suggest that periodontal disease would also consist of a factor associated with prematurity. Genetic and environmental factors are associated with this disease, but the microorganisms of the dental biofilm are vital in this process (Grigoriadou et al., 2010; Standar et al., 2010; Barros et al., 2014).
Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and other bacteria may be present in the periodontium and are unique to the oral cavity, and may disseminate to other sites of the body, reinforcing the oral focal infection thesis (Slots and Kamma, 2001). Such microorganisms would have the capacity to induce the formation of substances that would require an immune and inflammatory response of the host, cytokine production and other chemical mediators (Engeretson et al., 2000), who would have an important participation in the initiation of labor, being considered biologically plausible mechanisms that can connect the two conditions (Offenbacher et al., 1996, Dasanayake, 1998; Slots e kamma, 2001; Araújo et al., 2006; Zanatta et al., 2007; Mc Nicol e Israels, 2010).

Porphyromonas gingivalis is a bacterium frequently detected in pregnant women (Basaravaju et al., 2012) being identified at sites far from the mouth. It has several virulence factors, among these a variety of enzymes produced in its metabolism (Bainbridge et al., 2010). Such enzymes are released in the environment where the microorganism is installed, they manage the cellular signaling pathways, ensuring that it remains in the intracellular environment.

Aggregatibacter actinomycetemcomitans has been frequently detected in the gingival sulcus of the general population with aggressive periodontitis, mainly due to expression of the leukotoxin, which has specific destruction capacity of neutrophils and macrophages, acting extremely on the immune response of the host (Kachalany, 2010; Goulart et al., 2010; Brage et al., 2011; Claesson et al., 2011; Raslan et al., 2011). The detection of the microorganisms of the periodontium in the placenta and/or its annexes has been elaborated to establish the relation, considering the hematogenous route as an important possibility, where the invasion would occur through maternal blood, via fetal-placental circulation.

Placenta is considered a representation of an intimate parabiotic union of the maternal and fetal tissues, developing a complexity of functions, among them, homeostasis of maternal-fetal interchanges, production of hormones and integration of the hormonal requirements of the two organisms. In cases of infection, it is suggested that the main function of the organ would be the physical barrier, which postpones, but does not impede the transit of pathogens to the product of conception (Barros et al., 2014).

The use and utility of diagnostic methods in the identification and quantification of these periodontopathogens has been sought. The techniques of molecular biology are currently being proposed, allowing not only to acquire knowledge of microbial genetics, but also to lay the foundations for the development of diagnostic techniques (Bedran et al., 2010).

Based on these premises, the present study aimed to detect the expression of specific transcripts from the leukotoxin of Aggregatibacter actinomycetemcomitans and the RNA polymerase of Porphyromonas gingivalis, by means of a more functional molecular diagnosis that is the Reverse Transcription-PCR, using total RNA samples from placental tissue of mothers with periodontal disease.

Materials and methods

This study included women who had a preterm birth at the UFMS University Hospital (n = 19), Campo Grande - Mato Grosso do Sul, Brazil, from January 2013 to April
2014. The inclusion criteria were: (1) Women in preterm labor, ≤ 36 weeks gestation; (2) Presence of teeth in the four quadrants of the dental arch; (3) Free and Informed Consent Form (Protocol No. 2273 CAAE -0345.0.049.000-11) signed by the parturient (or guardian, when minors). Exclusions were made only in the case of failure to obtain consent. Control samples (n = 10) of mRNA from normal placentas were kindly donated by A. M. Miglioli, Department of Pediatrics UFMS / MS-Brazil. Briefly, placental tissue samples (300mg) were collected immediately after delivery, in duplicates, placed in micro tubes containing 500µl of RNA solution - RNA stabilization solution (Ambion) and stored in a freezer at -86°C until the procedure of RNA and DNA extraction.

Extraction of nucleic acids and qPCR

For the extraction of total RNA, 300 mg of placental tissue was used in 1 ml of Stat-60®, according to the protocol described by the manufacturer, and genomic DNA from the placenta samples were obtained by extracting the organic phase from the first stage of the RNA extraction by the same method.

The extraction of total RNA and DNase treatment of the samples was performed to verify the active presence of Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis, through expression of leukotoxin and RNA polymerase mRNAs, respectively. The target amplicons were designed and selected from the analysis of mRNA sequences described in Gene Bank using the NCBI Blastn program for the determination and synthesis of primers, sense and anti-sense oligonucleotides of approximately 21-24 nt containing ~ 50% GC. The primers and sizes of the target amplicons are shown in Table 1.

**Table 1** - Selected oligonucleotides, size (nt) and target fragment (bp), for the detection of Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis through qPCR, Campo Grande-MS, 2014

<table>
<thead>
<tr>
<th>PRIMERS</th>
<th>SEQUENCES</th>
<th>SIZE (nt)</th>
<th>AMPLICONS (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACOMLEUKFOR</td>
<td>5’-CTA GG T ATT GCG AAA CAA TTT G-3’</td>
<td>22</td>
<td>254</td>
</tr>
<tr>
<td>ACOMLEUKREV</td>
<td>5’-CCT GAA ATT AAG CTG GTA ATC-3’</td>
<td>21</td>
<td>254</td>
</tr>
<tr>
<td>PGINGFOR01</td>
<td>5’- GAT GCA TAA CGA CGA GCT TCT CGT -3’</td>
<td>24</td>
<td>183</td>
</tr>
<tr>
<td>PGINGREV02</td>
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<td>24</td>
<td>183</td>
</tr>
<tr>
<td>PGINGREV03</td>
<td>5’- ACG TTA TCT GCG GTC ACC TCA TTC -3’</td>
<td>24</td>
<td>72 (01+03)</td>
</tr>
</tbody>
</table>

(nt) – nucleotide, (bp) –base pairs.

Primers were originally designed in our laboratory, through the RNA sequences available from Genebank (NCBI nucleotide blast search: https://blast.ncbi.nlm.nih.gov). Lyophilized oligos, obtained from Integrated DNA Technology (https://www.idtdna.com), were resuspended in sterile filtered water and aliquoted for...
storage at -20°C in stocks of 100 pmol / µL. Primer solutions for use were diluted to 10 pmol / µL. The normalizer endogenous control used for the human S26 ribosomal RNA target gene (hss26for = 5'-tgt gcttcctgctgtatgtgaa -3' and hss26 rev = 5'-cga ttctg act acctgctgtg -3'; 75bp target fragment) was previously standardized in our lab NUFIGEN / ICB-UFMG.

The cDNA samples resulting from RT (scDNA = single stranded complementary DNA, was performed from 700 ng of total RNA in final reaction volume of 21 µL per sample), were used in PCRtr.

The qPCR was performed on the PRISM® 7500 / ABI Sequence Detection System using the reaction protocol described by the SYBR Green PCR Master Mix Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The samples were applied in 384-well plates (ABI PRISM® 384-Well Optical Reaction Plate with Barcode, Invitrogen Life Technologies, Carlsbad, CA, USA) in a final reaction volume of 10 µL each. Sample template aliquots of 0.8 µL were pipetted into each channel of the plate, and 9.2 µL of Mix sybr (5 µL of the SYBR Green PCR Master Mix Kit, 0.06 µL of each primer and antisense at 10 pmol / µl) and filtered sterile water (qsp 10 µl). The plate was sealed with optical adhesive (ABI PRISM Optical Adhesive Covers, Invitrogen Life Technologies, Carlsbad, CA, USA) in real time, occurred in the following thermal cycle: [stage 1] one cycle of 50 ° C / 2 min; [stage 2] one cycle at 95 ° C / 10 min; [stage 3] 40 cycles of 95 ° C / 0.15 min. For confirmation of fragment sizes amplified by Real-Time PCR, dissociation curves as part of the program established in PRISM® 7500 / ABI and amplified fragments were further visualized by nitrate-stained polyacrylamide gel electrophoresis (Carvalho et al., 2000).

Statistical analysis

For the relative quantification a comparative analysis of the expression of the transcripts of the target genes with the endogenous control was carried out, using the comparative CT method, where the endogenous control was used to normalize the expression of the target genes (mean CT target gene - mean of the endogenous control CT) generating ΔCT. Using the ΔCT, the ΔΔCT (ΔCT sample - ΔCT of the calibrator (reference sample) was calculated. The 2^ΔΔCT formula was then applied to determine the relative expression levels of each target gene.

Results

Figure 1 shows the typical results obtained by RT-qPCR from placental total RNA. The respective dissociation curves on the right characterize the specificity of the primers. The result of the relative expression of target genes per sample is shown in Figure 2.
Figure 1 - Typical results obtained by RT-qPCR. The amplification curves of the target fragments for human S26, Leukotoxin from Aggregatibacter actinomycetemcomitans and RNA polymerase from Porphyromonas gingivalis, respectively top to bottom left, from placental total RNA. The respective dissociation curves are on the right and characterize the specificity of the primers.
Figure 2 - Expression of mRNAs for Leukotoxin from Aggregatibacter Actinomycetemcomitans and RNA polymerase from Porphyromonas gingivalis, by RT-qPCR. (A) Comparison of mRNA expression for Leukotoxin among placental samples positive for the presence of Aggregatibacter Actinomycetemcomitans. (B) Comparison of mRNA expression for the bacterial RNA polymerase enzyme of Porphyromonas gingivalis. The bars represent the relative expression values obtained by the statistical method of $2^{-\Delta\Delta Ct}$ after correction with the endogenous normalizing RNA S26.

PCR from patients’ placental DNA detected $94.1\%$ (16/17) positives for Porphyromonas and $17.6\%$ (3/17) for Aggregatibacter, respectively. The reverse transcription qPCR detected $31\%$ (5/16) of Leukotoxin transcript expression from Aggregatibacter, and $75\%$ (12/16) showed RNA polymerase transcripts from Porphyromonas gingivalis (Table 2).
Table 2 - Results per sample, detection of genomic DNA by conventional PCR and expression of mRNAs encoding leukotoxin from Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis RNA polymerase, from total placental tissue RNA, by RT-qPCR.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Aggregatibacter Leukotoxin gene by Conventional PCR</th>
<th>Leukotoxin in mRNA expression by RT-qPCR</th>
<th>Porphyromonas RNApol by conventional PCR</th>
<th>PorphyromonasRNApol mRNA expression</th>
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<td>Total</td>
<td>+ 03</td>
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<td>Percentual</td>
<td>17,6</td>
<td>31,0</td>
<td>94,1</td>
<td>75,0</td>
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(+ positive  (N) negative  (#) not aplicable.

Discussion

The periodontopathogens studied here have virulence factors that make them unique. Aggregatibacter actinomycetemcomitans presents the leukotoxin that has the specific destruction capacity of neutrophils and macrophages, acting extremely well on the immune response of the host (Kachalany, 2010; Goulart et al., 2010, Claesson et al. Raslan et al., 2011). Porphyromonas gingivalis, on the other hand, presents a variety of enzymes produced in its metabolism that when released in the environment where the microorganism is installed, manage the cell signaling pathways, guaranteeing its permanence in the intracellular environment (Bainbridge et al., 2010).

It is important to contextualize that most current research involving the identification of periodontopathogens has been used to detect the DNA of the microorganism through qPCR using specific primers (Basaravaju et al., 2012; Swati et al., 2012). PCR alone, allows the identification of the pathogen, which differs from the presentation in the
present work, which in addition to detection of genomic DNA by conventional qPCR, we endeavored to verify the bacterial activity and its viability, considered here by the authors, as a functional diagnosis, which instigates the question of the virulence of these microorganisms and the capacity of their infection in placenta.

The presence of *Aggregatibacter actinomycetemcomitans* at sites far from the mouth by PCR has shown low percentages when the placenta and/or placental tissue have been investigated (Swati et al., 2012). In this aspect, it is important to note that in the present work, through the conventional real-time PCR technique, bacterial genomic DNA of *P. gingivalis* was detected in 84.2% of the samples. For *Aggregatibacter actinomycetemcomitans* these percentages were lower 15.8%, but both results are of diagnostic importance and application in more complete epidemiological studies. From the positive samples for genomic DNA, a significant confirmation was obtained in this research, that is, 31% of the placentas evaluated showed leukotoxin mRNA transcripts from *Aggregatibacter actinomycetemcomitans*, while 75% presented mRNA expression for RNA Polymerase of *Porphyromonas gingivalis*. These findings suggest that, in the PCR positive placentas, for these microorganisms, there is a large percentage of viable bacteria, that is, expressing proteins relevant for their proliferation in that tissue, with preponderance of *Porphyromonas gingivalis*.

On the other hand, *Porphyromonas gingivalis* is a bacterium frequently detected in pregnant women (Basaravaju et al., 2012). Researches using this microorganism used oral and placental tissue samples to obtain positivity for these microorganisms in blood and placenta, regardless of the status of periodontal disease, and had *Porphyromonas gingivalis* as the most expressive (Swati et al., 2012).

The presence of *Porphyromonas gingivalis* has also been identified in the amniotic fluid of women with preterm birth with intact membranes (Leon et al., 2007) and in puerperal placenta with poor reproductive outcome (Bearfield et al., 2002; Han et al. 2006; Barak et al., 2007; Feitosa, 2011). However, its definitive role for preterm birth has been debated. In this line, studies aimed at finding antibodies specific for *Porphyromonas gingivalis* were performed, the authors concluded that this microorganism can colonize placental tissue, and contribute to premature delivery, but the fact that the antibody reactivity of the microorganism has been established, does not confirm the presence of whole bacteria, but of their antigens (Katz et al., 2009).

Bacteremia are expected events when we consider periodontal disease, and these would contribute to this outcome, since they are frequent and recurrent with *Porphyromonas gingivalis* and other oral pathogens during the chewing process itself (Mao et al., 2007; Mc Nicol and Israels, 2011), a fact that could lead to the conjecture of the transit of this bacterium to the placenta, through the route of hematogenous dissemination. It is believed that the main function of the organ is that of physical barrier, which postpones, but does not impede the transit of pathogens in the conception process (Barros et al., 2014).

The invasion of the placental tissue is related to the significant virulence factors of the bacteria, which would cause perturbation of the homeostasis (Katz et al., 2009), deregulation of immune responses, by interrupting the expression of cytokines and increasing cell proliferation, suppressing apoptosis (Mao et al., 2007). There was also an increase in the activity of lipopolysaccharides and the activation of the synthesis of PGE2 and TNF-alpha by chorioamniotic cells and subsequent induction of preterm labor (Engebretson et al., 2000; Pio, 2008, Sert et al., 2011, Ribeiro, 2013).
The ability to withstand oxidative stress, which is submitted to the microorganism, is also another factor to be considered to characterize the uniqueness of *Porphyromonas gingivalis*, which has this property, which certainly facilitates its response in adaptation and is an important factor of virulence of this bacterium (Mysak et al., 2014). It is known that microbial species interact and may influence the disease process, favoring its growth or increasing the virulence potential of other microorganisms that may be present (Barros et al., 2014). The bacterial genotype and the load, genetic factors and host physiology are currently considered as of interest to study the colonization of *Porphyromonas gingivalis* in the placenta (Katz et al., 2009).

A review study on *Porphyromonas gingivalis*, presented a mechanism of adhesion to the host cell, observing that it is internalized by the membrane (lipid rafts), incorporating through the first phagosomes, activating the cellular autophagy to provide a favorable environment to replication, while suppressing apoptosis. The replicate vacuole contains the host cell proteins that were acquired by autophagy, contributing to the mechanism of survival of the pathogen (Mysak et al., 2014; Barros et al., 2014).

The reverse transcription and real-time PCR methodology has the same intense and specific amplification process, so that the specific transcript of the bacterium under study can be detected by the power of this technique and provides the information that the microorganism is not only present in the specimen studied, but also shows that the bacterium is alive and viable, but we know that traditional culture methods, such as blood culture, would not in most cases be able to present sufficient growth for an effective diagnosis for the treatment of the newborn patient with prenatal infection. The use of this molecular approach may be followed in the future by using other methodologies that demonstrate the presence and viability of microorganisms, such as western blot and immunofluorescence and confocal microscopies, however, we know that the amount of proteins expressed in these conditions is extremely rare for immunoblotting.

In conclusion, the functional diagnosis proposed in this work led to the finding that there was activity of the two bacteria in the placenta, with a greater detection of *Porphyromonas gingivalis*. This data is of great clinical importance, since the presence of *Porphyromonas gingivalis* in the placenta and its functional expression, evidences that the microorganism, which is of oral origin, had the capacity of translocation, suggesting here, an evidence of the relation between periodontal disease of the mother, and the prematurity of the newborn.

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Porphyromonas gingivalis induced platelet activation.


