Detection of Immunoglobulin G Against Oral Streptococci in Peripheral and Umbilical Cord Blood

Detecção de Imunoglobulina G Contra Streptococos Orais em Sangue Periférico e do Cordão Umbilical

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Abstract

The maternal Immunoglobulin G (IgG) antibodies transferred during the intrauterine life represents the main newborn immune protection against several intestinal and respiratory infection. However, there is little information about the reactivity and function of IgG from cord blood against newborn oral colonization. The aims this study was to evaluate the presence of IgG against Streptococcus mitis, Streptococcus mutans and their main virulence antigens (GbpB, Ag I/II and Gtf) in peripheral blood (PB) samples from mothers with S. mutans or not in the saliva (SA) and compare with umbilical cord blood (CB). PB and SA samples were obtained from healthy women in the Hospital admission and CB was collected after delivery. The specificity of IgG against the bacterial extracts was determined by Western blot. The genetic material detection of S. mutans in the salivas was realized by the quantitative polymerase chain reaction with specific primers. The results showed that the majority of blood samples showed IgG against S. mitis. On the other hand, the majority of samples exhibited IgG anti S. mutans. The number of reactive bands to S. mutans was significantly higher than S. mitis in both blood samples (p<0.05). The IgG anti-GbpB detection was more frequent than IgG anti-Ag I/II or anti-Gtf (p<0.05). The IgG antibody response pattern to S. mutans was similar in PB and CB pairs. There was no difference in the IgG anti-S. mutans detection and its virulence Ags (p>0.05) in the PB from mothers colonized or not by S. mutans. In opposite, in CB sample, it was more frequent to find samples with IgG anti-S. mutans and GbpB in the salivas from mothers with detectable S. mutans (p<0.05). In conclusion, the blood samples possessed IgG-anti S. mutans and theirs virulência antigens; mainly IgG anti-GbpB. The lack of IgG against S. mitis transferred by CB can be related to the early S. mitis colonization in the first months of age. The similarity of bands recognized by IgG against S. mutans antigens detected in PB and CB is suggestive that maternal antibodies can be transferred to the fetus.

Keywords: Streptococcus mutans. Saliva. Fetal Blood. Immunoglobulin G.

Resumo

Os anticorpos Imunoglobulina G (IgG) transferidos durante a vida intrauterina representam a principal proteção imunológica do recém-nascido contra diversas infecções intestinais e respiratórias. No entanto, poucas informações existem sobre a reatividade e função de IgG do sangue do cordão contra a colonização oral de recém-nascidos. Os objetivos deste estudo foram avaliar a presença de IgG contra Streptococcus mitis, Streptococcus mutans e seus principais antígenos de virulência (GbpB, Ag I/II e Gtf) em amostras de sangue periférico (SP) de mães com S. mutans detectável ou não na saliva (SA) e comparar com sangue do cordão umbilical (SC). Amostras de SA e SP foram obtidas de mulheres saudáveis na admissão do hospital e CB foi coletado após o parto. A especificidade de IgG contra os extratos bacterianos foi determinada por Western blot. A detecção do material genético de S. mutans nas salivas foi realizada pela reação em cadeia da polimerase quantitativa com primers específicos. Os resultados mostraram que a maioria das amostras de sangue apresentou IgG contra S. mitis. Por outro lado, a maioria das amostras exibiu IgG anti S. mutans. O número de bandas reativas para S. mutans foi significativamente maior do que contra S. mitis em ambas as amostras de sangue (p<0.05). A detecção de IgG anti-GbpB foi mais frequente do que IgG anti-Ag I/II ou anti-Gtf (p<0.05). O padrão de resposta de anticorpos IgG contra S. mutans foi semelhante nos pares SP e SC. Não houve diferença na detecção de IgG anti-S. mutans e sua virulência Ags (p>0.05) no SP de mães colonizadas ou não por S. mutans. Por outro lado, nas amostras SC, foi mais frequente encontrar amostras com IgG anti-S. mutans e GbpB em salivas de mães com S. mutans detectáveis (p<0.05). Em conclusão, as amostras de sangue possuíam IgG-anti S. mutans e seus antígenos de virulência; principalmente IgG anti-GbpB. A falta de IgG contra S. mitis transferida no SC pode estar relacionada a colonização precoce de S. mitis nos primeiros meses de idade. A semelhança de bandas reconhecidas por IgG contra antígenos de S. mutans detectados em SP e SC é sugestiva de que anticorpos maternos podem ser transferidos para o feto.

Palavras-chave: Streptococcus mutans. Saliva. Sangue Fetal. Immunoglobulina G.

1 Introduction

Streptococci correspond to the majority of commensal bacteria that firstly colonize the oral cavity. Streptococcus mitis is the main bacteria that initially colonize the oral cavity. After tooth eruption, new colonization sites arise and the oral microbiota becomes progressively more complex. The presence of non-desquamating dental surfaces is sufficient for colonization by Streptococcus mutans. However, these species may also be detected in predentate children, in children highly exposed to saccharose consumption and to contact with the saliva of highly infected individuals. A high colonization with S. mutans is a risk for oral health since S. mutans is considered to be the main primary etiological agent of dental caries. Caries is a great public health problem...
especially in Brazil, where the levels of early caries are high, with evidence that infants aged 5 to 11 months already show high *S. mutans* levels detectable in the saliva\(^7\). Mothers seem to be the main sources of *S. mutans* infection in children\(^1\). In the first 48 hours of life, the newborn gains a major part of his or her oral microflora from his or her mother\(^6\).

*S. mutans* ability to adhere and to accumulate on smooth surfaces forming dental biofilms, and to metabolize carbohydrates and survive in low pH environments represent key factors of its virulence mechanisms. This microorganism produces glycosyltransferases (Gtfs) that may utilize saccharose to produce glucans, which are glucose exopolymers that, when combined with glucan-binding proteins (Gbps), favor the cariogenic streptococci cohesion and agglutination on the dental surface\(^7\). The past studies support the use of these antigens in clinical trials of caries vaccines in older children and adults\(^8\).

The mucosa immune system represents the first line of the adaptive immune response defense against oral infectious challenges. The secretions antibacterial activity is mediated in part by the immunoglobulin A (IgA) present in the saliva. Complex pattern of salivary IgA reactivity to *S. mutans* antigens\(^9\) was found in heavily exposed children aged 5-24 months, suggesting that responses to virulence-associated antigens, especially against GbpB, may influence the *S. mutans* ability to colonize the oral cavity. At birth, babies have low levels of salivary IgA antibodies against the virulence antibodies of *S. mutans*, such as Ag I/II, Gtf and GbpB, especially in premature infants, demonstrating that the repertoire of saliva IgA may not be able to combat the initial oral microbial colonization\(^10\).

Besides IgA, human saliva have IgG transported to the oral cavity, via gingival crevicular fluid, mucosal transudate and ultrafiltration through the salivary gland acini\(^11\). There is a lack of data in the literature regarding the transport of immunoglobulins against *S. mutans*. Neonatal immunity depends on this intrauterine acquisition of IgG through active transplacental transport mediated by FcRn receptor\(^12\). Immunoglobulin G, transferred during the intrauterine life, represents the main newborn immune protection and may contribute to the bacterial neutralization and exclusion processes and the establishment of intestinal microbiota\(^13\). The levels of transport in singleton pregnancies depend on gestational age, maternal IgG levels and IgG subclass\(^14\). Several studies have reported the specific IgG detection against various microbial types, which is of protective importance at the beginning of life and effective for formation and transfer to the fetus after antigen stimulation of pregnant woman. Specific IgG against *Pseudomonas, Klebsiella, group B streptococci*\(^15\) and *Staphylococcus aureus*\(^16\) was detected in umbilical cord.

The presence of IgG anti-*S. mutans* and its virulence antigens was found in human serum\(^17,18\) and saliva\(^19\). However, the IgG role in the *S. mutans* installation and accumulation processes is still controversial. IgG present in the gingival fluid can inhibit the *S. mutans* adhesion, neutralizing the enzymes, promoting the opsonization and consequent phagocytosis and/or activation of the complement system\(^20\). Serum IgG antibodies to *S. mutans* cell-wall associated protein antigens were found more frequently in caries-free subjects\(^17\) and low caries experience has been associated with significantly higher serum IgG\(^18,21\). On the other hand, an association between IgG and caries levels\(^22\) was not detected and serum IgG and IgA levels were increased in patients with caries\(^20\).

Therefore, there is little information about the IgG immune response during the early stages of *S. mutans* bacterial challenge and the IgG role provided by umbilical cord blood against the installation of these bacteria. It is possible that some factors can affect the initial response of pioneer commensal species of the oral cavity, and influence the immune response patterns and the susceptibility to colonization. In the present study, the aims were to analyze the presence and specificity of IgG against oral streptococci and compared the reactivity of IgG to *S. mutans* and *S. mitis* extracts in umbilical cord blood (CB) and peripheral blood (PB) samples of mothers with detectable levels of *S. mutans* in the salivas samples (SA).

2 Material and Methods

2.1 Study design

Eighty pairs of pregnant women were enrolled in this study, under consent for their participation. The Ethical Committee of the Medicine School of Ribeirao Preto, Sao Paulo, Brazil, (protocol number 13290/2010), approved this study. To be included in the study population, health mothers were selected and submitted to cesarean delivery of full-term gestation without antibiotic therapy. Information about maternal and gestational background was obtained by interviewing the expectant mother on the first day of hospitalization. The exclusion criteria included placental and congenital malformation, chronic disease, and infectious disease during pregnancy. A subset of 33 mothers had the saliva collected to investigate the presence of genetic material of *S. mutans*.

2.2 Samples collections

The peripheral blood (PB) and salivas samples were collected from mothers in the first day of hospitalization. After delivery, the blood was drawn from the umbilical vein using a sterile needle and syringe. Whole saliva samples unstimulated were collected using sterile polypropylene transfer pipettes. Solution of 250 mM EDTA, pH 5.2 (Sigma, St Louis, MO, USA) was added to each sample prior to transport on ice to the laboratory where they were stored at -80 °C until analysis.

2.3 *S. mutans* detection in the salivas samples

Firstly, the samples were incubated with lysozyme
solution (Sigma, Tokyo, Japan) and then, genetic material was extracted with the PowerLyzer PowerSoil DNA Isolation Kit (MO-BIO, Carlsbad, CA) according to the manufacturer’s instructions. The concentration of purified DNA product was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific). Sm479F/R primer pair (Sm479F: 5’-TGGCGAAAAGATAAAACAAAC-3’ and Sm479R: 5’-GGCCCTCTGATATGGTCTTAG-3’) that is highly specific and sensitive for identification of S. mutans in mixed DNA samples (23) purchased from Invitrogen (Tokyo, Japan).

The StepOne™ Real-Time PCR System (Thermo Fisher Scientific) performed PCR. Each reaction tube contained reaction mixture, including 6.5 µL SYBR Green Master Mix (Roche, Ilhois, USA), 1µL of each primer, 4.5µL de ultrapure water e 2 µL of DNA extracted from the samples. The cycling conditions were 45 cycles of 15 s at 94 °C for denaturation, 30 s at 56 °C for annealing, and 30 s at 72 °C for extension followed by a melting curve analysis of the PCR product. Two PCR reactions were performed for all the samples. To positive control DNA from Streptococcus mutans was used (UA159).

2.4 IgG reactivity against streptococci antigens

Patterns of IgG antibody reactivity against S. mutans (UA159), S. mitis (ATCC506), Ags were determined in Western blot as previously described (9). As negative controls, membranes were incubated only with blocking buffer, and as positive controls; membranes were incubated with a standard saliva sample obtained from an adult whose reaction pattern with antigen extracts had been previously measured. The secondary antibody was goat IgG anti-human IgG conjugated with horseradish peroxidase (Sigma, 1: 4,000 dilutions). Antibody reactions were developed using an ECL system (GE Healthcare UK, Little Chalfont, United Kingdom). For this purpose, immunoblots were incubated with ECL detection J Health Sci 2020;22(4):270-5 solution and tested by ANOVA. A determined and compared between antigens and samples number of IgG-reactive bands in Ags extracts was also determined and compared between antigens and samples.

The developed X-ray films were scanned in a scanning densitometer (GE Healthcare) to analyze antigen recognition patterns, including the number of reactive bands. A film blank value was subtracted from the reactive band value.

2.5 Statistical analysis

A chi-square and Fisher’s Exact Test tested comparisons of the frequencies of IgG antibody specificities. The mean number of IgG-reactive bands in Ags extracts was also determined and compared between antigens and samples and tested by ANOVA. A p-value of <0.05 was considered statistically significant.

3 Results and Discussion

3.1 Frequency of IgG reactivity to S. mutans and S. mitis extracts in the samples

The results show that IgG anti-S. mitis was found in a minority of blood samples (less than 22%) (Table 1). On the other hand, the immunoassays showed that more than 95% of blood samples had IgG anti-S. mutans (Table 1). Comparative analysis of the bacterial strains revealed that S. mutans was more recognized than S. mitis both in PB and CB (p<0.05).

There was no difference in the frequency of presence of IgG anti-S. mutans between the blood samples (Tab. 1, p>0.05). Immunoglobulin IgG represents systemic immunity and is present in whole saliva through the gingival fluid and can be associated to caries control. A large amount of IgG antibodies against S. mutans were transferred from mother to child, as also detected for other species. The findings of this study agree with previous data reported by Luo et al. who also detected antibodies against these oral streptococcal species. The repertory of IgG antibodies transferred to the fetus through the umbilical cord depends on the mother’s contact with the antigen diversity to which she is exposed, explaining the less frequent detection and number of bands of S. mitis reactive IgG compared to S. mutans, since S. mitis is a microorganism frequently detected in infants during the first months of life and practically absent in adults.

There was no significant difference in number of reactive bands between PB and CB for the two bacterial species (Table 1, p>0.05). Comparative analysis of S. mutans and S. mitis showed that the mean number of S. mutans IgG-reactive bands was significantly greater than for S. mitis extract in both PB and CB (p>0.05).

Table 1 - Frequency of samples with IgG reactive to bacteria extracts and Mean number of reactive bands in the samples with a positive response to Streptococcus mutans and Streptococcus mitis antigens in PB (Peripheral Blood) and CB (Cord Blood) samples

| Bacterial Extracts | SM | SMI |
|--------------------|----|-----|
| CB | PB | CB | PB |
| Frequency of samples with IgG reactive to bacteria extracts (%) | 76 (95) | 78 (98) | 21 (26) | 22 (28) |
| Mean (± SD) number of reactive bands | 4.10 ± 2.34 | 4.71 ± 2.38 | 1.52 ± 0.81 | 1.68 ± 0.94 |

Source: Research data.

3.2 Frequency of IgG reactivity against S. mutans antigens

Data regarding the specificity of the presence of IgG reactive to the virulence antigens of S. mutans (Ag I/II, GTF and GbpB) in PB (n=78) and CB (n=76) samples are presented in Table 2. Most PB and CB samples presented IgG against GbpB, while IgG positivity against Ag I/II was detected in about half the samples (Table 2) and Gtf was reactive in 29 and 41% of the CB and PB samples, respectively. There was no difference in frequency of IgG reactive to antigens between PB and CB (Table 2, p>0.05).

The results revealed a high frequency of samples with IgG reactive to GbpB, since more than 89% of the blood samples (PB and CB) contained IgG reactive to this protein, whereas...
50% and 35% of the samples contained IgG reactive to Ag I/II and Gtf, respectively. The presence of high concentration of IgG anti-GbpB was also detected by Chia et al.\textsuperscript{17}

However, there was a significant difference in the detection frequency of Ag I/II- or Gtf-specific IgG compared to GbpB for both PB and CB (Table 2, p<0.05) since the IgG reactive to GbpB was more frequent than the other antigens. There was also a difference between Ag I/II and Gtf in the frequency of a positive and negative response in CB since there was a higher prevalence of positivity for Ag I/II than for Gtf (Table 2, p<0.05). There was a positive correlation in the frequency of IgG reactive or not to Ag I/II, GTF and GbpB between PB and CB (p<0.05, r=0.71).

### Table 2 - Frequency and percentage of positive (Yes) and negative (No) responses to the Streptococcus mutans virulence antigens (Ag I/II, GTF and GbpB) in PB (n=78) and CB (n=76) samples

| S. mutans Ags: | Number (%) of samples with reactive IgG | | | | | |
|---------------|--------------------------------------|---|---|---|---|
|               | PB (n=78)                            | CB (n=76)                      |
|               | Yes     | No     | Yes     | No     |
| Ag I/II       | 45 (58)\textsuperscript{a}          | 33 (42)\textsuperscript{a}   | 37 (49)\textsuperscript{a} | 39 (51)\textsuperscript{b,d} |
| Gtf           | 32 (41)\textsuperscript{b}          | 46 (59)\textsuperscript{b}   | 22 (29)\textsuperscript{b} | 54(71)\textsuperscript{a}  |
| GbpB          | 71 (91)\textsuperscript{a,b}        | 7 (9)\textsuperscript{a,b}   | 68 (89)\textsuperscript{b,c} | 8(11)\textsuperscript{b}   |

Superscript letters indicate statistically significant differences considering p<0.05, \textsuperscript{a,b} Chi-square test, p<0.05, q=22.76, \textsuperscript{b,c} Chi-square test, p<0.05, q=43.46, \textsuperscript{a,b} Chi-square test, p<0.05, q=6.23, \textsuperscript{a,b} Chi-square test, p<0.05, q=29.59, \textsuperscript{a,b} Chi-square test, p<0.05, q=57.64.

Source: Research data.

These maternal IgG antibodies in the newborn circulation start to decay during the first 3 months of life since they are metabolized and are accompanied by a gradual increase of the levels of the baby’s own antibodies\textsuperscript{25}. In newborns, the salivary IgA may not be able to combat the initial oral colonization because they have low levels of IgA and its virulence antigens may be compensated for IgG transferred during pregnancy and specific IgA with avidity for S. mutans, while 70% of non-colonized mothers had this antibody (Table 3, p<0.05). Also a significant difference was found in CB regarding the specific response to GbpB, with 95% of the CB samples of colonized mothers containing IgG reactive to GbpB, compared to 62% of non-colonized mothers (Table 3, p<0.05).

No difference was detected between the frequency of samples with IgG reactivity to S. mutans and its antigens in PB and positive or negative bacteria detection in the saliva. However, in CB samples, 100% of the mothers carrying S. mutans in the saliva had IgG against S. mutans and 95% had GbpB reactive IgG, differing from the pregnant women with undetectable S. mutans.

### Table 3 - Frequency of saliva samples positive for the detection of Streptococcus mutans and IgG reactivity to Streptococcus mutans and its antigens in PB and CB

| IgG against | Number (%) of samples with reactive IgG | | | | | |
|-------------|--------------------------------------|---|---|---|---|
|              | Colonized N=20                      | Non-Colonized N=13 | | | |
|              | +       | -      | +    | -      |
| S. mutans total extract | | | | |
| PB          | 20 (100.0)     | 0 (0.0) | 12 (59.2) | 1 (7.7) |
| CB          | 20 (100.0)     | 0 (0.0) | 9 (69.2)  | 4 (30.8) |
| Ag I/II     | 13 (65.0)      | 7 (35.0) | 6 (46.2)  | 7 (53.8) |
| Gtf         | 11 (55.0)      | 9 (45.0) | 4 (30.8)  | 9 (69.2) |
| CB          | 8 (40.0)       | 12 (60.0) | 2 (15.4)  | 11 (64.6) |
| GbpB        | 19 (95.0)      | 1 (5.0)  | 10 (76.9) | 3 (23.1) |

Superscript letters indicate statistically significant differences considering p<0.05

Source: Research data.

S. mutans is known to be a common microorganism in the oral microbiota associated with the dental caries formation, but it causes a multifactorial disease in which a person harboring the bacterium in his or her oral cavity does not always have active disease. S. mutans may also be a transitory microorganism in the oral cavity, explaining the lack of S. mutans detection in the saliva of some puerperal who had reactive IgG in PB. Mothers highly colonized by S. mutans may offer a large quantity of IgG antibodies against S. mutans simultaneously, as observed in 50% of the PB samples and 49% of the CB samples.

### 3.3 Comparison of the frequency of IgG-anti S. mutans and its antigens with the presence of genetic material of S. mutans in maternal saliva

Sixty percent of mothers’ salivas were positive for the S. mutans detection. No significant differences were detected between frequency of IgG-anti S. mutans and genetic material detection of S. mutans (Table 3, p>0.05). However, some differences were found in CB in comparison with S. mutans detection in the saliva. All samples of colonized mothers contained IgG anti-S. mutans, while 70% of non-colonized mothers had this antibody (Table 3, p<0.05). Also a significant difference was found in CB regarding the specific response to GbpB, with 95% of the CB samples of colonized mothers containing IgG reactive to GbpB, compared to 62% of non-colonized mothers (Table 3, p<0.05).

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to the neonate\textsuperscript{11}. Maternal \textit{Staphylococcus aureus} nasal colonization at delivery also was not associated with higher antibody levels in the newborns’ mother \textsuperscript{18}. The degree of similarity in PB and CB regarding IgG specificity against SM and its virulence antigens was high since more than 85% of the pairs of samples showed the same response profile. These results support the possibility of immunoglobulins transference against \textit{S. mutans} in the gestational period especially from the 30th to 34th week of gestation, when the antibodies passage is more intensified.

4 Conclusion

This study revealed that the majority of blood samples presented IgG against \textit{S. mutans}, especially against GbpB. There is a similarity in IgG reactive to \textit{S. mutans} and their virulence antigens in the CB and PB. The \textit{S. mutans} detection in the oral cavity is associated to IgG presence in the samples.

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