



Molecular analysis of oral pathogens and saliva, dental caries

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Lecture topics

- Factors involved in development of dental caries:

- Oral microbiome
 - Dental plaque (bacterial biofilm)
- Saliva
 - Saliva composition and its functions
- Behavioral and environmental factors
- Genetic factors

tissue susceptibility to caries

- Molecular analysis of saliva
- Molecular analysis of oral microbiome
- Genetic basis of dental caries
 - Genetic association studies related to dental caries

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Dental caries and factors affecting their development



(www.betterhealth.vic.gov.au)

Dental caries

- the most common disease

– 32 % of the world population



- shares risk factors with many other non-communicable diseases
- however \rightarrow Streptococcus mutans transmission from parents to infants (Early childhood caries)

– complex disease

- multifactorial (endogenous and exogenous factors), multiple genes are involved in

- interaction of several factors contributes to formation of caries

- genetic predispositions
- composition of oral microflora
- composition and physical action of saliva
- overall health condition (immune system disorders, systemic diseases affecting immune system)
- behavioral and environmental factors
- time for which the factors act / interact



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Dental caries

- caries dentium



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- enamel \rightarrow 95 – 98 % of inorganic matter (apatites - the most important mineral component) apatite – cationic complexes = ligands Ca²⁺ and (PO₄)²⁻

- counter-ions → Ca₁₀(PO₄)₆CO₃ (*carbonate apatite*), Ca₁₀(PO₄)₆(OH)₂ (*hydroxyapatite*), Ca₁₀(PO₄)₆F₂ (*fluoroapatite*) dynamic process characterized by alternating periods of demineralization and remineralization of the enamel → ↑ demineralization → → carious lesions progression

 – caries formation → organic acids (bacteria, diet) dissolve the mineral part of the enamel by neutralizing apatite counter-ions (crystal structure disintegration)

 \rightarrow proteolytic enzymes \rightarrow degradation of organic matrix (collagens and proteoglycans)

- carious lesion \rightarrow dentin \rightarrow dentinal tubules \rightarrow pulp \rightarrow pulpitis, periodontitis



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Color Atlas of Biochemistry (3rd edition, 2013)

- Saliva:

- complex carioprotective factor
- maintaining homeostasis
- physical effect

saliva flow (washing and lubrication of tissues of oral cavity),

Acid Tooth ename (pH < 5,5)Hydroxyapatite luorapatite (PO.).F

Remineralization (F⁻ ion presence)

Tooth ename

рH

6 - 7

HPO.²

dentalcare.com/en-us/professional-education/ce purses/ce410/fluoride-s-mechanism-of-actio

Hydroxyapatite

(PO,) (OH)

Fluorapatite

Ca. (PO.) F.

oral cavity clearance (washing of harmful substances, not adhered microorganisms)

components

gustin (Carbonic anhydrase VI \rightarrow buffering capacity), calcium, phosphate, fluoride ions, proteins of specific (IgA, IgG) and non-specific immunity (lysozyme, defensins), other components of immune system

- $-\downarrow$ food remnants, \downarrow microorganisms, \downarrow acidity (dilution, buffer systems bicarbonate, hydrogen phosphate, proteins), \uparrow substances with antibacterial, antifungal and antiviral properties, \uparrow balance between re- and demineralization
- reduced saliva flow \leftarrow dehydration, obstruction / hypofunction of salivary glands (complex) diseases), medicaments (beta blockers, antidepressants, antihistamines), drugs (methamphetamine, THC)
 - \rightarrow promotion of carious lesions formation



- Oral microbiome:

 second largest and diverse (over 700 species of bacteria) homeostasis maintenance (competition and displacement of exogenous pathogens → maintaining ecosystem stability), immunomodulation

– dental plaque = biofilm =

matrix of extracellular polymeric substances (EPS)

+ aerobic bacteria (*Streptococcus sanguinis*), facultative anaerobes (*S. mutans, S. sobrinus, Lactobacillus* sp.), anaerobes (*Actinomyces* sp., *Veillonella* sp.), fungi (*Candida* sp.)

- saliva → proteins with charged surfaces (acidic PRPs, statherin, histatins) → electrostatic interaction with phosphate and calcium ions of apatite
- → (acquired) pellicle formation (mucins, cystatins, albumin, IgA, IgG, Iysozyme, alpha-amylase, carbohydrates, neutral lipids, phospho- and glycolipids, glucosyltransferase)
 - \rightarrow protection against demineralization, partial reduction of microbial adhesion
 - \rightarrow substrate for bacteria \rightarrow biofilm formation \rightarrow dental plaque



Early colonizers: S. mutans: Late colornizers: mainly health-associated streptococci glucan production acid-tolerant (e.g. S. sanguinis and S. gordonii) robust biofilm formation acid-producing acid-tolerant (e.g. Lactobacillus and Veillonella spp.) acid-producing glucan Poor oral hygiene High-sugar diet Other salivary, immunological and microbial factors

– Dental plaque:

 problem: <u>dysbiosis</u> of oral microbiome disruption of homeostasis

tps://doi.org/10.3389/fmicb.2018.03323

→ ↑ cariogenic species (ferment carbohydrates to organic acids + tolerate low pH environment)
 → prevailing Streptococcus mutans a Streptococcus sobrinus, Lactobacillus sp., Candida sp.

- factors promoting cariogenic species

 \downarrow saliva, \downarrow oral hygiene \rightarrow \uparrow plaque thickness; \uparrow intake of sugars / acids \rightarrow acidification; \downarrow immunity, inflammation,...

↑ dental plaque → lack of oxygen → ↑ anaerobic metabolism → metabolism of fermentable carbohydrates → organic acids $\rightarrow \downarrow$ pH → demineralization $\rightarrow \rightarrow \rightarrow$ caries

S. mutans \rightarrow dextran (α -1,6-D-glucan) \rightarrow extracellular insoluble polysaccharide \rightarrow \uparrow protection of bacteria against adverse environment (low pH, antimicrobial factors), \uparrow co-adhesion of other species, \uparrow plaque adhesion

– External factors:

poor oral hygiene

poor eating habits (excessive intake of fermentable carbohydrates)

smoking

alcohol consumption

medicaments (salivary glands function impairment, acidification of oral cavity, antibiotics)

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poor access to quality food, drinking water, hygiene supplies, medical care

– Time

– Genetic predisposition:

- complex disease (genetic, epigenetic and exogenous factors)

- multiple genes
- genetic heterogeneity locus heterogeneity (mutations in genes at different loci), allelic heterogeneity (different mutations at the same locus)
- incomplete penetrance pathological phenotype is not manifested in all individuals carrying disease-causing gene (positive effects of other alleles or exogenous factors)
- phenocopy pathological phenotype is manifested by individuals who are not carrying disease-causing gene
- high frequency of risk alleles in population
- ethnic variability (disease-causing genes can vary among populations, variant alleles can have different impact on phenotype in different populations)

 \rightarrow it is possible to determine only genes (alleles) which act as risk factors \rightarrow predisposition (predisposing genotype can increase probability of disease development, but does not determine the disease)

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Molecular analysis of saliva

Saliva as a Diagnostic Fluid



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- Saliva as a diagnostic fluid

- saliva collection: non-invasive, easy, painless, repeatable (available material), can be used for all age categories
- markers of diseases of the oral cavity, systemic diseases (disease diagnostics, monitoring of disease development depending on therapy)
- salivaomics uses high-throughput technologies (genomics, transcriptomics, proteomics, metabolomics, lipidomics and microbiomics) to study saliva components and identify biomarkers

Table describing examples of commonly analyzed biomarkers in whole mouth saliva; CRP – C-reactive protein; HPLC – high performance liquid chromatography; IC – ion chromatography; LC-MS – liquid chromatography mass spectrometry; MALDI-TOF MS matrix assisted laser desorption ionization-time of flight mass spectrometry; RT-LAMP – reverse transcriptase loop-mediated isothermal amplification; AOPP – Advanced Oxidation Protein Products; TBARS – Thiobarbituric Acid Reactive Substances; TAC – Total Antioxidant Capacity; FRAS – Free Radical Analytical Systém.

Group of molecules	Biomarkers	Method			
Cytokines	Interleukins Tumor-necrotising factor Interferons, Chemokines	Multiplex array Luminex fluoresce technique			
Acute phase proteins	CRP	ELISA			
Inflammatory proteins	Myeloperoxidase Neutrophil elastase	ELISA			
Antibodies	Anti-HIV, Anti-RO Anti-La	RT-LAMP ELISA			
Hormones	Testosterone Estradiol Cortisol	ELISA HPLC			
Enzymes	Amylase Lysozyme	ELISA MALDI-TOF MS			
Proteins-polypeptides	Immunoglobulin A Lactoferrin	MALDI-TOF MS ELISA			
Nucleic acids	DNA methylation DNA mutations microbiome	Microarray Sequencing			
Vitamins	25(OH)D(3) vitamins A, C, E	LP-MC ELISA			
lons	Na+, K+, Mg2+, Cl-, Ca2+, NH3-	IC			
Oxidative stress	AOPP TBARS	Spectrophoto- Spectrofluorometric methods ELISA			
Antioxidant status	TAC FRAS	Spectrophoto- Spectrofluorometric methods ELISA			

Janšáková et at., Klin. Biochem. Metab., 26 (47), 2018, No. 1, p. 21–26

- Saliva as a diagnostic fluid

- worse reproducibility of results \leftarrow high variability
 - technical (sampling, processing, method used)
 - interindividual
 - biological (influence of the condition of the oral cavity, systemic diseases (Sjögren's syndrome), xerostomia (medicaments),
 - ↓↓↓ analyte concentration in comparison with serum → ↑ saliva sample volume, detection limit, depletion of abundant proteins

\rightarrow standardization

- \rightarrow correlation of protein markers to saliva total protein concentration
- → standardization of used methods

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Antibodies	Anti-HIV, Anti-RO Anti-La	RT-LAMP ELISA			
Hormones	Testosterone Estradiol Cortisol	ELISA HPLC			
Enzymes	Amylase Lysozyme	ELISA MALDI-TOF MS			
Proteins-polypeptides	Immunoglobulin A Lactoferrin	MALDI-TOF MS ELISA			
Nucleic acids	DNA methylation DNA mutations microbiome	Microarray Sequencing			
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- Saliva as a diagnostic fluid dental caries susceptibility
 - point-of-care testing (chair-side diagnostic kits):
 - physical properties: volume, flow rate, viscosity, consistency
 - pH and buffering capacity of saliva

– lactate

- commercial kits (visual or colorimetric detection)
- commercial kits (colorimetric detection)
- determination of cariogenic bacteria S. mutans and Lactobacillus sp.

commercial kits (immunochromatographic detection of antigen, cultivation kit)

- Saliva as a diagnostic fluid - dental caries susceptibility

- saliva protein biomarkers associated with caries susceptibility:

↑ saliva total protein, total antioxidant capacity (TAC)
 ↑ alpha-amylase, mucins (MUC1 a MUC5B)

↓ arginine deiminase system, albumin, proteinase 3, PRP1/3, statherin, histatin 1
 ↓ concentrations of calcium and bicarbonate ions
 ↓ urease activity

- saliva protein biomarkers associated with susceptibility to early childhood caries:

↑ PRPs, histatins, IgA, IgG

↓ statherin

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– Saliva as a diagnostic fluid – examples of disease biomarkers

- autoimmune diseases

Sjögren's syndrome - α -amylase, carbonic anhydrase VI, lactoferrin, β 2-microglobulin

neurodegenerative diseases

Alzheimer's disease - total tau protein, phosphorylated tau protein, amyloid-β and alpha-synuclein

genetic diseases

cystic fibrosis – Ca, PO_4^{2-} , Na, K, Cl, \downarrow saliva volume, urea, uric acid, prostaglandin E_2

– cancer

<u>squamous cell carcinoma</u> – IL-8, IL-6, IL-1β, IL-4, IL-1, VEGF, HER2, tissue polypeptide antigen (TPA) and EGFR, LDH, N-α-acetyltransferase 10 protein (Naa10p), carcinoembryonic antigen (CEA) protein, serum basic fibroblast growth factor (bFGF), transferrin, cyclin D, Maspin, specific mRNAs

<u>breast cancer</u> - HER2/neu (C-erbB-2), VEGF, EGF, specific mRNAs, autoantibodies against HER2 and MUC-1 <u>pankreas cancer</u> – transcriptomic markers of mRNAs (*KRAS, MBD3L2, ACRV1* a *DPM1*), specific miRNA, lactoperoxidase, Cyclophilin B, Cytokeratins (14, 16 a 17)

 $M \vdash D$

endocrine diseases

Cushing's syndrome and Addison's disease - cortisol

sex hormones - polycystic ovary syndrome, menopause / andropause, anovulation, hypogonadism, hyperestrogenism

– Saliva as a diagnostic fluid – examples of disease biomarkers

- cardiovascular diseases

CK-MB, myoglobin, troponin I, myeloperoxidase, inflammation markers (CRP, TNF-α, MMP-9), cellular adhesion molecules (soluble CD40 and ICAM-1)

metabolism

diabetes mellitus type 2 - 1,5-anhydroglucitol, CRP, leptin, IL-6, TNF-α

infectious diseases

HIV - antibodies against HIV

viruses - IgM / IgA antibodies, viral RNA

Candidiasis, amebiasis - Candida sp., Entamoeba histolytica (antibodies)

Hepatitis - DNA of HBV virus

Peptic ulcer disease, gastritis - Helicobacter pylori (IgG antibodies, H. pylori DNA)

allergy

food allergies - IgE and IgG_1

periodontitis

bacteria of ,red complex' (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*), *Aggregatibacter actinomycetemcomitans* biomarkers - MMP-8, MMP-9, osteoprotegerin, ALT, α-amylase

Saliva as a diagnostic fluid diseases biomarkers



https://www.oraldna.com/trends-in-salivary-testing/

Test (Manufacturer)	Intended Use			
23andMe® Health + Ancestry	Detect genetic health risks (e.g., BRCA1/BCRA2 status), carrier status, physical traits, and wellness features			
Alert 2™ (OralDNA Labs)	Combine MyPerioPath® and MyPerio ID® IL-6			
Celsus One™ (OralDNA Labs)	Evaluate genetic markers related to inflammatory response			
DNA DrugMap™ (OralDNA Labs)	Detect drug metabolizer status			
Intercept [®] i2™, Intercept [®] i2he™, and Intercept® Oral Fluid Drug Test (OraSure Technologies, Inc.)	Detect drugs of abuse (e.g., marijuana, cocaine and opiates)			
MyPerio ID $^{\textcircled{8}}$ IL-6 or IL-1 (OralDNA Labs)	Detect genetic polymorphisms associated with increased genetic risk for severe periodontal disease Evaluate the number and concentration of bacteria implicated in periodontitis Detect CD44 and total protein associated with oral cancer			
MyPerioPath [®] (OralDNA Labs)				
OraMark™ Test (Vigilant Biosciences)				
OraQuick [®] In-Home HIV Test, OraQuick [®] HIV Self Test, and OraQuick ADVANCE [®] Rapid HIV-1/2 Antibody Test (OraSure Technologies, Inc.)	Detect HIV-1 and/or HIV-2 antibodies in oral fluid			
OraRisk HPV [®] (OralDNA Labs)	Screening tool to identify the type(s of oral HPV present			
OraRisk HSV [®] (OralDNA Labs)	Detect HSV-1 or HSV-2 present in the oral cavity			
OraRisk [®] Candida (OralDNA Labs)	Detect and identifies all common species of <i>Candida</i> present in the oral cavity			
OraRisk [®] CT/NG (OralDNA Labs)	Detect the presence of <i>Chlamydia</i> <i>trachomatis</i> and/or <i>Neisseria</i> <i>gonorrhea</i> in the oropharynx			
OraSure [®] HIV-1 (OraSure Technologies, Inc.)	Detect HIV-1 antibodies in oral fluid			
Q.E.D. Saliva Alcohol Test (OraSure Technologies, Inc.)	Detect alcohol in oral fluid			
SaliMark OSCC [®] (PeriRx, LLC)	Detect increased levels of certain mRNAs associated with increased			

risk of oral cancer

https://www.ada.org/en/member-center/oral-health-topics/salivary-diagnostic

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Molecular analysis of oral microbiome



https://www.jorthodsci.org/viewimage.asp?img= JOrthodontSci_2014_3_4_125_143233_f6.jpg

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- Oral microbiome
 - biomarker of dental caries and periodontitis
 - polymicrobial infection \rightarrow microbiological profile of patients

 \rightarrow disease susceptibility

 \rightarrow early diagnosis of disease (before onset of symptoms)



Microbial colonization occurs on all available surfaces, and microorganisms can also penetrate epithelial tissues and cells. The microbiota assembles into biofilm communities on the abiotic and biotic surfaces. In health (left), eubiotic biofilms maintain a homeostatic balance with the host. In disease (right), caries and periodontitis ensue when biofilms become dysbiotic, resulting in increased levels and duration of low pH challenge and the induction of destructive inflammatory responses, respectively. EPS, extracellular polymeric substance; GCF, gingival crevicular fluid.

https://doi.org/10.1038/s41579-018-0089-x

→ monitoring the course of disease and effectiveness of treatment (shift of microbiota from dysbiosis to eubiosis), targeted treatment

- \rightarrow an effective tool for disease prevention (evidence of patient dental care)
- \rightarrow development of new therapeutic approaches, personalized dental treatment

→ research - an effort to fully characterize a "healthy" microbiome (Which components of the microbiome should be monitored to evaluate the return of the microbiome from dysbiosis to a state compatible with health? Is it sufficient to monitor only selected key species or is it necessary to use multispecies assays?)

- Oral microbiome - methods of analysis

- point-of-care testing (chair-side diagnostics) :

dental caries risk – device CariScreen Susceptibility Testing Meter (Oral BioTech LLC) – after wiping the plaque off the tooth surface by special brush a reaction of ATP occurs nad bioluminescence is measured by the device \rightarrow bacterial activity *S. mutans*

<u>analysis in laboratory</u>

commercial kits - an example

periodontitis – kit MyPerioPath[®] (OralDNA Lab), saliva test, testing presence and amount of 11 bacteria species that promote periodontitis onset and development (quantitative real-time PCR analysis)





 $M \vdash D$



- Oral microbiome - methods of analysis

- sampling

→ microbial communities present on different parts of the oral cavity (saliva, tongue, palate, buccal mucosa, tooth surfaces, gums, supra- / subgingival plaque, tonsils, throat) show an overall similarity, but with small differences

 \rightarrow sample collecting from a specific site / rinsing of the whole mouth

- Oral microbiome - methods of analysis

- microbiological cultivations

 \rightarrow OM is one of the most complex microbial communities in the human body \rightarrow some species have not yet been cultivated

- 16S rRNA sequencing

 \rightarrow sequencing of the conserved gene for 16S rRNA, the most common method, taxonomic data only

- whole genome shotgun sequencing (WGS)

→ DNA is randomly cut and then subjected to Sanger sequencing or NGS; a tool for metagenomic analysis; not only taxonomic data but also biological functional profiles of the microbial community

- <u>qPCR</u>

→ not only the gene for 16S rRNA, but also other genes; also allows quantification

– ELISA test

 \rightarrow antigens, *P. gingivalis*

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- Oral microbiome



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@ Genomic Trees > taxa. The eHOMD site offers easy to use tools for viewing all publicly available ADT bacterial genomes. Welcome! 2017-11-22 01:11 Genome Annotation Update - @ Download Genomic Data Primary Investigators: Tsute Chen, Floyd E. Dewhirst, Isabel Fernandez Escapa, Yanmei Huang, Katherin P. Lemon, Bruce J. Paster, and William G. Wade 2016-02.27 16.92 2016-02.27 16.92 @ Download All Protein Sequences Annotated from Genomes Current Research Contributors: Erica Prosdocimi, Hayley Thompson, Nezar Al-hebshi, and Prasad Gajare. 2016-02.26 17.92 2016-02.26 17.92 @ MOND Jools Past Research Contributors: Oxana Baranova, Jessica Blanton, Anuj Carmanocha, Derrick Fouts, Akila Ganesan, Jacques Izard, Taylor Joyce, Alice Kirega, Erin Klein, Abby Lakshmanan, Cori Leonetti, Maoxuan Lin, Emmanuel Mongodin, Alexandra Rybalka, Derek Spencer, Annot Tanner, Sonia Vartoukian, Griffin Weigle, Larry Yang, and Wen-Han Yu Image Interfere Spencer, Annot Tanner, Sonia Vartoukian, Griffin Weigle, Larry Yang, and Wen-Han Yu Image Interfere Spencer, Annot Tanner, Sonia Vartoukian, Griffin Weigle, Larry Yang, and Wen-Han Yu Image Interfere Spencer, Annot Tanner, Sonia Vartoukian, Griffin Weigle, Larry Yang, and Wen-Han Yu Image Interfere Spencer, Annot Tanner, Sonia Vartoukian, Griffin Weigle, Larry Yang, and Wen-Han Yu Image Interfere Spencer, Annot Tanner, Sonia Vartoukian, Griffin Weigle, Larry Yang, and Wen-Han Yu Image Interfere Spencer, Annot Tanner, Sonia Vartoukian, Griffin Weigle, Larry Yang, and Wen-Han Yu Image Interfere Spencer, Annot Tanner, Sonia Vartoukian, Griffin Weigle, Larry Yang, and Wen-Han Yu Image Interfere Spen	HOMD JBrowse Genome Viewer »	representatives (Chlorobi, Chloroflexi, GN02, TM7, SR1, WPPS-2. Hence, the total number of genomes are 2087 including non-oral/non-r	asal	1		
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dysbiosis as a noninvasive biomarker

- Oral microbiome

 a potential biomarker of systemic diseases



Oral and systemic diseases associated with the oral microbiome. A representation of the associations found between diseases with increases or decreases of the abundances of organisms in the oral cavity. Organisms listed in blue have been shown to be increased in abundance in the oral cavity in individuals presenting with the noted disease, and organisms listed in red have been shown to be decreased. Those in purple may be either increased or decreased depending on the conditions or progression of the disease.

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Genetic association studies - candidate gene approach (case-control studies)

Case-control study for genetic association



http://www.discoveryandinnovation.com/BIOL20 2/notes/lecture25.html

- Candidate genes

- Selection of suitable candidate genes

in general, based of known biological, physiological and/or functional relevance to the disease

search for new potential genes (alleles) in the whole genome (GWAS, QTL - quantitative trait locus)

- <u>Suitable candidate genes for caries association studies</u>

genes participating in tooth development and affecting its morphology genes related to immune response genes related to production and composition of saliva genes related to taste preferences

- Candidate genes

- Selection of alleles (polymorphisms)

SNP, CNV, VNTR

based on studies already performed in other populations, GWAS, QTL

minor allele frequency is sufficient in a given population $(\downarrow \text{ frequency of allele in the population } \leftarrow \uparrow \text{ number of cases / controls})$

linkage disequilibrium among SNPs \rightarrow tagSNP

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- Methods of genotyping

- selection of an appropriate methodical approach

number of polymorphisms to be determined

total number of samples to be genotyped

quality of DNA sample (genomic DNA - blood, saliva, buccal swab)

costs - equipment, chemicals, consumables

availability of commercial genotyping services

Comparison of methods used for mannose-binding lectin gene (MBL2) genotyping.

	Allele -Specific PCR (AS-PCR)	ARMS ³ /Double ARMS ³ (+ Multiplex Allele -Specific PCR)	PCR and Restriction- Fragment Length Polymorphism (PCR-RFLP)	Commercial TaqMan Assay ⁵	High-Resolution Melt Analysis (HRMA)	Commercial INNO-LiPA MBL2 kit (Reverse PCR-SSOP)	Руго- Sequencing	Sanger Sequencing	MBL2 SNaPshot Assay
principle of allele discrimination/detection	PCR with a primer specific for one allele	PCR with primers specific for both alleles	allele-specific enzymatic cleavage of PCR amplicon	allele-specific hybridization of fluorescently labelled probe	temperature-dependent allele-specific hybridization of fluorescently labelled probe	hybridization of biotinylated PCR product with membrane immobilized sequence-specific oligonucleotide probes	chemiluminiscence-based detection of nucleotides during sequencing-by-synthesis reaction	detection of the sequence of an oligonucleotide amplified in PCR with fluorescently labelled dideoxyribonucleotides	allele-specific SBE by a single fluorescently labelled dideoxyribonucleotide (minisequencing)
post-PCR analysis	yes	yes	yes	no	no, when real-time PCR thermocycler is used	yes	no	yes	yes
analysis time	2 h ²	2-3 h ²	2 h + 1–3 h ⁴	1–2 h ⁶	1–1.5 h + 2–8 min. ⁸	3-4 h	2-3 h	6–7 h	56 h
number of work steps	2 (PCR, gel analysis)	2 (PCR, gel analysis)	4 (PCR, gel analysis, RFLP, gel analysis)	l (real-time PCR)	l (when real-time PCR thermocycler is used for PCR and subsequent melting temperature analysis)	9 (PCR, gel analysis, denaturation, hybridization, 2 washing steps, 3-step color development)	4 (PCR, gel analysis, purification, pyrosequencing)	5 (PCR, enzymatic cleaning, sequencing reaction, purification, analysis on sequencer)	5 (PCR, enzymatic cleaning, SBE reaction, enzymatic cleaning, analysis on sequencer)
automatic analysis	no	no	no	yes	yes	no	yes	yes	yes
number of analyses for complete $MBL2$ haplogenotype 1	12	6	6	6	5	1	4 9	2	1
number of oligonucleotide primers + labelled primers/probes for complete <i>MBL2</i> haplogenotype ¹	24 primers	15 primers	6 primers	6 TaqMan assays (12 primers + 12 TaqMan probes)	10 primers + 5 TaqMan probes	4 primers	8 primers + 4 biotinylated primers	2 primers	8 primers
estimated cost of analysis of whole haplogenotype ¹	1 USD	1 USD	2 USD	2 USD	1 USD	product was discontinued	2 USD	5 USD	1.50 USD
input amount of template DNA	20–200 ng	20–200 ng	50–500 ng	1-20 ng	10–20 ng	200–500 ng	10–100 ng	10-250 ng	10-100 ng
assay robustness	low	low	low-medium	medium-high	high	low-medium	medium	medium-high	medium-high
special equipment requirement	-			real-time PCR thermocycler	real-time PCR thermocycler or fluorescence scanning/detection system	water bath with shaking platform, aspiration apparatus	vacuum prep workstation, pyrosequencing machine	automated DNA sequencer	automated DNA sequencer
SNP genotyping throughput	low	low	low	high	high	medium	high	high	high
software for automatic allele calling	по	no	no	yes (SDS software, SNPman program) ⁷	yes real-time PCR instruments with HRMA compatible software with genotype auto-calling function	по	no	yes (Mutation Surveyor, GeneMarker, Minor Variant Finder Software, SeqScape™ Software, Variant Reporter™ Software) 10	yes (GeneMapper, GeneMarker) ¹¹
Ref.	[41]	[30,42]	[43,44]	[27]	[28]	[29]	[45]	[37]	-

¹ rs11003125, rs7096206, rs7095891, rs5030737, rs1800450 and rs1800451; ² Analysis time depends on polymerase chain reaction (PCR) length and gel concentration; ³ ARMS-amplification refractory mutation system; ⁴ Separation time depends on gel concentration and the length of cleaved fragments; ⁵ TaqMan[®] SNP Genotyping Assays (Applied Biosystems⁵), C_2336609_20 (rs1800451); ⁶ depends on number of cycles; ⁷ Sequence Detection Software (SDS) by Applied Biosystems⁵⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragments; ⁶ TaqMan[®] SNP Genotyping Assays (Applied Biosystems⁵⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragments; ⁵ TaqMan[®] SNP Genotyping Assays (Applied Biosystems⁵⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragment; ⁵ TaqMan[®] SNP Genotyping Assays (Applied Biosystems⁵⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragment; ⁵ TaqMan[®] SNP Genotyping Assays (Applied Biosystems⁵⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragment; ⁵ TaqMan[®] SNP Genotyping Assays (Applied Biosystems⁵⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragment; ⁵ Software by Applied Biosystems⁵⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragment; ⁶ SoftGenetics⁶ (https://software by Applied Biosystems⁵⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragment; ⁶ SoftGenetics⁶ (https://software by Applied Biosystems⁷⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragment; ⁶ SoftGenetics⁶ (https://software by Applied Biosystems⁷⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragment; ⁶ SoftGenetics⁶ (https://software by Applied Biosystems⁷⁴ (www.thermofisher.com (accessed on 2 November 2020)), SNP: near qostrate fragment; ⁶ SoftGenetics⁶ (https://software by Applied

- Genotyping methods

– PCR+RFLP (restriction fragment lenght polymorphism)

 \rightarrow PCR amplification followed by specific restriction digestion



Huang, BCM Cancer (2008)

- Genotyping methods

– allele-specific PCR



https://doi.org/10.1016/j.jim.2004.10.007

 $M \vdash 1$

- \rightarrow primers that are specific for particular allele
- \rightarrow if the allele is present \rightarrow amplification product is generated \rightarrow detection



5'agaaaatgettacceaggeaageetgtgtaaaacacea-308-teactgeeaeggaaageatgtttatagtetteeageageaeg3' 3'tettttacgaatgggteegtteggacaeattttgtggt-308-agtgaeggtgeetttegtaeaaatateagaaggtegtegttge5'



- Genotyping methods

real-time PCR → fluorescently labeled hybridization probes
 →commercial TaqMan probes



homozygote 3.000 1.500 0.000 0.00 6.00 12.00 18.00 24.00 30.00 30.00 30.00 30.00 30.00 24.00 30.00

VIC





- Genotyping methods
 - Sanger sequencing \rightarrow sequence of a part of DNA with polymorphism



- Genotyping methods

Single Nucleotide Polymorphism
 Detection with the iPLEX® Assay
 and the MassARRAY® System



 $M \vdash D$

Genes that have been associated with increased risk of dental carries

- Proteins involved in development of enamel

AMELX – Amelogenin gene

ENAM – Enamelin gene

TUFT1 – Tuftelin gene

KLK4 – gene encoding Kallikrein-related peptidase 4

AMBN – Ameloblastin gene

TFIP11 – gene encoding Tuftelin-interacting protein 11

MMPs (MMP20) – genes encoding Matrix Metalloproteinases



Schematic diagram of histological changes in amelogenesis. The histological development of enamel crystals goes hand in hand with changes in ameloblast morphology. Undifferentiated epithelial cells receive signals to transform into secretory ameloblast cells of some 75 µm tall and ~5 µm in diameter with a specialized distal cell process (Tomes' process) which plays an important role in matrix exocytosis. These same cells will retransform into shorter cells (~35 µm tall) during maturation devoid of the Tomes' process. In maturation stage, ameloblasts undergo cyclical changes from a cell with a distal ruffled border, the ruffled-ameloblast (RA), to a cell with a smooth distal border, the smooth-ameloblast (SA). Tight junctions are found at the basal and apical pole of secretory ameloblasts. The apical or distal pole is closest to the enamel crystals. In RA cells, tight junctions are found only at the apical pole but in SA cells they are located at the basal pole. Organellar distribution differs in cells at each stage.

https://doi.org/10.1113/JP272775

 $M \vdash 1$

https://doi.org/10.1016/j.sjbs.2020.11.071

 Genes that have been associated with increased risk of dental carries

- Taste receptors \rightarrow associated with heightened preference for sweet taste $\rightarrow \uparrow$ sugar intake

TAS2R38 – gene encoding Taste receptor 2 member 38 → G protein-coupled receptor, responsible for sensitivity to bitter taste

TAS1R2 / TAS1R3 – genes encoding Taste receptor 1 member 2 and 3 → G protein-coupled heterodimeric receptor, responsible for sensitivity to sweet taste

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https://doi.org/10.1080/00016357.2020.1832253

Sugars:

sucrose, fructose

Sweet receptor:

T1R2/T1R3

Artificial sweeteners

saccharin, aspartame

Na*, K* entry through TRPM5

Depolarization

quinine, denatonium

salicin

Bitter receptor

Ca²

Na*, K* entry through TRPM5

Depolarization

[]/| |− |]

/doi.org/10.3390/s1004034

T2Rs

Absorption of carbohydrates



Glucose transporter \rightarrow associated with heightened preference for sweet taste $\rightarrow \uparrow$ sugar intake

- GLUT2 gene encoding Glucose transporter 2 required for glucose-stimulated insulin secretion (pancreatic β -cells), controls perception of glucose (nervous system) \rightarrow control of food intake
 - expression is required for the physiological control of glucose-sensitive genes, its inactivation in the liver leads to impaired glucose-stimulated insulin secretion

Genes that have been associated

with increased risk of dental carries

[]/| |− |]

Genes that have been associated with increased risk of dental carries

– Proteins of immune system

Schematic representation of the lectin pathway of the complement system. The lectin pathway (LP) is triggered by five pattern recognition receptors (PRR): mannose-binding lectin (MBL), ficolin-1, -2, and -3, and collectin 11 (CL11 or CL-K1). The LP is initiated when these PRRs bind to pathogen-associated molecular patterns (PAMPs) on the surface of pathogens or to apoptotic or necrotic cells (damage-associates molecular patterns, DMAPs). Circulating MBL, CL11, and ficolins form complexes with MASP-1 and MASP-2. After the binding of MBL, ficolins, and CL-11 to their targets, MASP-1 auto-activates and triggers MASP-2. Activated MASP-2 cleaves C4 and C2 allowing the assembly of the C3 (C4bC2a) and C5 (C4bC2a(C3),)convertases and the subsequent activation of the terminal pathway. Activated MASP-1 also cleaves C2. MAC = membrane attack complex.

https://doi.org/10.1080/08927014.2020.1856821

https://doi.org/10.1007/978-1-4614-9209-2_7-1

MBL2 – gene encoding Mannose-binding lectin (AKA Mannose-binding protein, Mannan-binding protein/lectin, Collectin 1, MBP1, or Mannose-binding protein C)

- soluble serum lectin recognizing specific carbohydrates on bacterial surfaces $\rightarrow \downarrow$ complement activation



– Genes that have been associated with increased risk of dental carries

- Proteins in saliva



https://doi.org/10.1590/1807-3107bor-2017.vol31.0041

DEFB1 – gene encoding β-Defensin 1 – an antimicrobial peptide from family of Defensins (alpha, beta), which includes cysteine-rich cyclic cationic peptides. They are part of innate immunity, create channels in the cytoplasmic membrane of bacteria, stimulate the immune system incl. complement (classical pathway), act as chemoattractants.

dental caries experience.

- LTF Lactoferrin gene transport globular glycoprotein, binds free iron. Part of innate immunity, antibacterial (peroxides are formed when interacting with bacterial membranes), antiviral (competition of adhesion of viral particles to host cells, binding to particles of certain types of viruses), antifungal (against *C. albicans*) activity, stimulation of phagocytosis.
- LYZL2 gene encoding Lysozyme-like protein 2 part of C-type Lysozyme family. Hydrolyzes glycosidic bonds in peptidoglycans (breaking down the cell wall of G⁺ bacteria).

- Genes that have been associated with increased risk of dental carries
 - Proteins in saliva



https://doi.org/10.1590/1807-3107bor-2017.vol31.0041

- CA6 gene encoding Carbonic anhydrase VI enzyme also called ,gustin', catalyzes the hydratation of carbon dioxide to form bicarbonate ions and protons. Saliva pH maintenance (bicarbonate buffer system)
- MUC7 gene encoding Mucin 7 low molecular weight glycoprotein (MG2), participates in the formation of acquired pellicle and thus in bacterial adhesion and biofilm formation
- *MUC5B* gene encoding Mucin 5B glycoprotein (MG2), participates in the formation of acquired pellicle and thus in bacterial adhesion and biofilm formation
- PRH1 gene encoding salivary acidic proline-rich phosphoprotein 1, participates in the formation of acquired pellicle and thus in bacterial adhesion and biofilm formation

– Pitfalls of genetic association studies of dental caries

- too many factors play a role in etiopathogenesis → set of patients (cases) can never be perfectly categorized → perfectly defined set of cases cannot be created → maximally defined set of cases as far as possible
- most studies do not confirm the association, only suggests (some studies even give conflicting results)

 \rightarrow further association studies (more samples) - studies of individual polymorphisms (but their effect may be small), but also genes and loci (gene-based and gene-cluster analysis) \rightarrow further strengthening of results

 \rightarrow meta-analysis – a combination of data obtained by an exhaustive search of published and unpublished data worldwide \rightarrow increasing the consistency of the results (by increasing the strength of the result). Many primary studies are too small to demonstrate an important clinical effect (not strong enough). A combination of all studies that answer the same clinical question \rightarrow increase in statistical power or significance level

 detailed questionnaires for evaluating the influence of psychological, sociological, economic and behavioral factors → fragmentation of set of cases to too many groups of too few patients



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