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The use of commercial ELISA kits in measuring CA6 concentration of saliva

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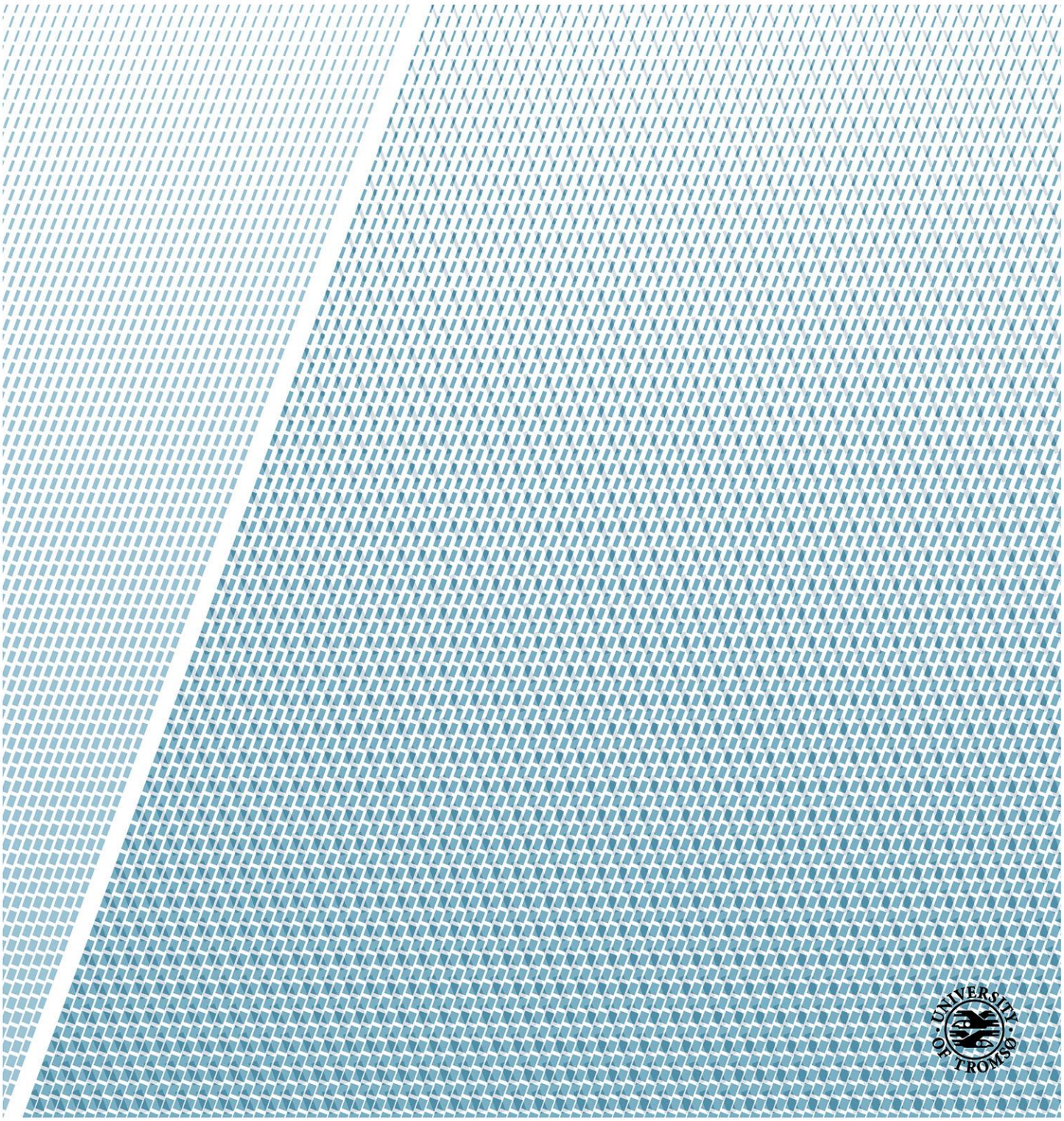

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Acknowledgement

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Keywords: CA6, ELISA.

Abstract

Introduction: Carbonic anhydrase 6 (CA6) is the only secreted member of the carbonic anhydrase enzyme family. This enzyme catalyzes the reversible hydration of carbon dioxide: $CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-$. The dehydration reaction contributes to net acidification in several tissues and biological fluids. Salivary CA6 attaches to the enamel and predisposes to caries probably by acidifying the dental biofilm by catalyzing carbon dioxide dehydration. Enzyme-linked immunosorbent assay (ELISA) is an assay technique commonly used to measure protein concentrations in solubilized samples. Commercial ELISA kits for specific antigens are available from several companies. The quality of an ELISA kit can vary in terms of sensitivity, specificity, detection range and intra-assay variation. Although commercial ELISA kits for CA6 have been used in several studies, the basic quality parameters of the kits have not been reported by independent research groups. The main objective of this study was to determine whether the two different commercially available ELISA kits are reliable for CA6 concentration measuring. We hypothesized that the two commercial ELISA kits are reliable for measuring CA6 concentration.

Material and Methods: We obtained two kits from two companies that we from now on call «Company X» and "Company Y". One experiment was run with the "Composite X" kit (competitive ELISA) and three experiments were run with the "Company Y" kits (sandwich ELISA) from three different batches (Batch 1, 2 and 3). For ELISA assay procedure, we followed the manufacturers' instructions. We collected saliva samples from the same two participants in all the experiments, and both of the samples was present in every experiment. In addition, we used purified CA6 which we received as a kind gift from Professor Seppo Parkkila's group (Tampere, Finland).

Results: None of the kits detected the purified CA6 (Table 2). Only the "Company X" kit and the batch 3 of "Company Y" kits gave a positive reaction in saliva samples.

Discussion: The findings of this study indicate that one should be vary of studies where commercial non-certified kits have been used. It might be that the results of these studies are based on unspecific binding. This is to our knowledge the only study examining the quality of commercial CA6 ELISA kits. The results obtained in this study indicate that quality assessment of commercial ELISA kits is necessary, at least for CA6 kits if not to other kits as well.

1. Introduction

1.1. Saliva and salivary glands

Salivary glands produce the saliva, which is secreted, through the salivary ducts, into the oral cavity. Salivary glands are exocrine glands i.e. they secrete fluid on to a free surface, in this case the oral cavity. There are minor and major salivary glands. The paired major salivary glands are the parotid, submandibular and sublingual glands. There are 450 to 1000 small salivary glands which are scattered throughout the oral mucosa (Berkovitz, Holland, Makdissi, & Moxham, 2017, pp. 303-316).

Even though saliva has 99% water, the additional 1% of proteins, inorganic compounds and lipids, allow the saliva to serve many roles. The viscosity of secreted saliva from one gland differs from secreted saliva from another gland. Some glands produce a watery (serous) saliva and others produce saliva that is a bit sticky (mucous). The parotid gland produces serous saliva, the sublingual gland produces mucous saliva, whereas the submandibular gland produces a mix between the two. The minor salivary glands mainly produce mucous saliva, except for the von Ebner glands which are serous. This difference in the viscosity is caused by mucoproteins. When the saliva from the different glands enters the oral cavity, it is as transparent as water. The saliva then gets mixed and contaminated with bacteria, food debris, shed mucosal cells and the gingival crevicular fluid. This fluid is mostly referred to as saliva, but a more correctly term is whole saliva. The whole saliva consists of a variety of different proteins which plays a part in protecting the tooth and preventing caries through different mechanisms. The main functions of the saliva are lubrication, solubilization, microbial defense, remineralization of tooth and initiation of starch digestion (Berkovitz et al., 2017, pp. 303-316; Fejerskov, Nyvad, & Kidd, 2015, pp. 83-90; Tiwari, 2011; Wang, Zhou, Li, & Zhang, 2019).

Saliva has been used in diagnostics for more than two thousand years (Tiwari, 2011). Some of the reasons as to why saliva makes such a good diagnostic tool is because the collection of saliva is non-invasive, fast, easy and inexpensive. During collection of saliva, no needles are needed, which makes it more comfortable than serum for patients. Saliva also reflects the body's physiological and pathological states (Lee & Wong, 2009).

One can collect either stimulated saliva or unstimulated/resting saliva. To get stimulated saliva one needs a stimulant, usually paraffin gum. To collect the unstimulated saliva one needs to drool in a cup with one's head leaning downwards (Gomar-Vercher, Simon-Soro, Montiel-Company, Almerich-Silla, & Mira, 2018). Determining saliva characteristics e.g. saliva flow, consistency, pH, buffer capacity and the presence of different enzymes and microorganisms, may aid in coming up with a diagnosis and prognosis. Some of the saliva characteristics are associated with caries experience (Chifor et al., 2019).

1.2. Dental caries

Dental caries is tooth decay resulting from bacteria metabolism end products. The term caries lesion is used to describe the results (signs and symptoms) of a localized chemical dissolution on the surface of the tooth. The lesions develop as a result of metabolic events in the dental biofilm covering the affected area of the tooth (Brusevold, 2020; Fejerskov et al., 2015, pp. 7-11).

The process of caries typically starts below the surface of the enamel and is a result of the demineralization of the tooth's crystalline mineral structure, the hydroxyapatite. The demineralization is caused by the organic acids produced by the bacteria in the dental biofilm as a result of sugar metabolism. The acids cause the pH to drop below the critical point for the hydroxyapatite (pH5.7) below which net dissolution of hydroxyapatite from the enamel occurs. When the pH rises over the critical point, remineralization occurs. During remineralization, the dissolved ions precipitate to the demineralized area of the tooth. The pH in the oral cavity changes during the day because of readily metabolized sugar ingestion, resulting in demineralization. The balance between remineralization and demineralization determines whether caries lesions develop or not (Fejerskov et al., 2015, pp. 155-160; Pitts et al., 2017)

The metabolic activities of bacteria in the dental biofilm results in caries. More than 1200 different types of bacteria have been detected in the mouth, but in order for bacteria to play a role in caries they need to have specific characteristics which promotes caries. These characteristics are the capability to transport fermentable sugars when competing with other plaque bacteria, and it must also have the ability to convert fermentable sugars to acid. The bacteria should be able to maintain the metabolism of sugar, even under low pH and other extreme environmental conditions, and produce extracellular polysaccharides. One species of

bacteria that is known to have all these characteristics is *Streptococcus Mutans*. It is considered to be the most cariogenic bacteria. Several cross-sectional and longitudinal studies have shown a strong, but not absolute, connection between the detection of a caries lesion and the amount of *Streptococcus Mutans* in the dental biofilm (Fejerskov et al., 2015, pp. 107-125).

The most important risk factor for caries is high intake of readily fermentable sugars. In a recent systematic review Moynihan (2016) reports a lowered caries risk in patients who have an intake of free sugars of $\leq$ 10% of energy (Moynihan, 2016).

A method often used to measure a person's caries experience is the DMFT/DMFS-index, which stands for decayed, missed, filled teeth/surfaces. By counting a person's number of decayed, missed, filled teeth/surfaces, one gets a person's DMFT/S-value. In caries epidemiology, this index is widely used because of its versatility, simplicity and amenability to statistical analysis, but the index also has its disadvantages. For example, in high-income countries where dental treatment is highly accessible, the F (filled) component dominates. On the other hand, in low-income countries where the access to dental treatment is limited, the D (decayed) component dominates (Fejerskov et al., 2015, pp. 24-26).

Globally, caries is affecting more than 35% of people of all ages (Pitts et al., 2017). In fact, caries is one of the most common health problems (Hegde, Attavar, Shetty, Hegde, & Hegde, 2019). Several factors together effect caries development i.e. caries is a multifactorial disease. These factors are mentioned in a causal model which is called the Keyes triad. The simple version of the Keyes triad describes the association between caries and the dental biofilm, pH and the tooth. This model was later expanded to include more biological factors. The new model is called the Fejerskov and Manji model for caries causation and includes other biological factors such as saliva flow rate and composition, buffer capacity, fluoride and microbial species. The Fejerskov and Manji model also takes a few social-behavioral factors such as social class, income, knowledge, attitudes, behavior and education into consideration. Time was also added into this model (Fejerskov et al., 2015, pp. 39-40).

As mentioned above, there are several factors that can affect the development of caries, and because of this several measures have been taken to control caries. Tooth brushing controls caries efficiently, especially in combination with fluoride toothpaste. In fact, the use of

fluorides in toothpastes is considered to be the main reason for the overall global reduction in caries over recent decades in many countries. Fluoride replaces the hydroxyl-group in the dental hydroxyapatite to form fluorapatite. It has a lower critical point than the hydroxyapatite, pH 4.5. Thus, fluorapatite precipitate to remineralize dissolve dental tissues more readily than hydroxyapatite. Moreover, fluorapatite withstands a more acidic environment than hydroxyapatite. Proper tooth brushing disturbs the dental biofilm maturation to acidogenic state (Fejerskov et al., 2015, pp. 245-250; Pitts et al., 2017).

Over the years, several studies have been conducted to on caries prediction. Among other things, salivary proteins have been considered an additional tool in caries prediction. One of the salivary proteins that has been studied is carbonic anhydrase 6 (CA6).

1.3. Carbonic anhydrase 6 (CA6)

Carbonic anhydrase 6 (CA6) is the only secreted member of the carbonic anhydrase enzyme family. This enzyme catalyzes the reversible hydration of carbon dioxide: $CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-$. The dehydration reaction contributes to net acidification in several tissues and biological fluids. CA6 is primarily expressed in the serous acinar cells and ductal cells of excretory glands, especially in the parotid and submandibular glands (Parkkila et al., 1990). CA6 secretion into the saliva has a circadian periodicity declining to a very low concentration during sleep, but waking up and meals have an obvious increasing effect on salivary CA6 concentration (Kivela, Parkkila, Parkkila, Leinonen, & Rajaniemi, 1999; Kivela et al., 1997; Parkkila, Parkkila, & Rajaniemi, 1995). In paraffin-stimulated saliva, the mean concentrations of CA6 is 6.8+/- 4.3mg/L, and the rate of secretion is 10.2 +/- 7.9µg/min (Parkkila, Parkkila, Vierjoki, Stahlberg, & Rajaniemi, 1993). The CA6 concentration show wide individual variation from 0.3 mg/L to 17 mg/L (Esberg, Haworth, Brunius, Lif Holgerson, & Johansson, 2019). However, the CA6 concentrations of saliva samples collected before and after breakfast show strong positive correlation (Kivela et al., 1997).

Some studies have found an association between high concentrations of CA6 and high caries incidence, while others have not found no association or have found an opposite association (low CA6 concentrations are associated with high caries experience) (Culp et al., 2011; Kivela, Parkkila, Parkkila, & Rajaniemi, 1999). One example is the study by Culp et al. (2011) on genetically modified CA6-deficient mice. The wild-type mice had higher caries

incidence than the CA6-deficient mice. Their results further indicated that salivary CA6 attaches to enamel and predisposes to caries probably by acidifying the dental biofilm by catalyzing carbon dioxide dehydration (Culp et al., 2011). In a recent pilot follow-up study, the participants with caries increment had higher salivary CA6 concentrations than the participants with no caries increment (Leinonen et al. unpublished results). On the other hand, results from a study by Kimoto et al. (2006) concluded that CA6 facilitates acid neutralization by salivary bicarbonate, and because of this may be considered an anti-caries protein (Kimoto, Kishino, Yura, & Ogawa, 2006). Also, a study by Kivelä et al. (1999) found a negative correlation between the values of salivary CA6 concentrations and the DMFT-value (Kivela, Parkkila, Parkkila, & Rajaniemi, 1999).

A study by Piekoszewska-Zietek et al. (2019) found no significant relations between salivary CA6 concentration and caries indices, except the active caries group, in which the concentrations of CA6 were significantly lower. The study used commercial ELISA kits from Cloud-Clone Corp. (Piekoszewska-Zietek, Szymanski, & Olczak-Kowalczyk, 2020).

Another study by Esberg et al. (2019) found that secreted amounts of CA6 (µg/min) but not concentration was significantly higher in caries-free than caries-affected 17 years old. In accordance, twice as many caries-free, compared to caries-affected, participants were identified in the highest tertile based on the distribution of secreted CA6 amounts with an odds ratio of 3.5 to be caries-free. In their study by Esberg et al. (2019) used Biosite ELISA kits to measure CA6 concentration in the saliva samples (Esberg et al., 2019).

A third study by Daniele C. R. Picco et al. (2019) found that CA6 concentration was significantly higher in the biofilm and saliva of caries-free children than in the saliva of children with caries. They used Cloud-Clone Corp. ELISA kits (Picco, Marangoni-Lopes, Parisotto, Mattos-Graner, & Nobre-Dos-Santos, 2019).

1.4. Competitive and sandwich ELISA.

Enzyme linked immunosorbent assay (ELISA) is a laboratory method to detect and quantify proteins such as CA6. ELISA can detect and quantify small amounts of antigens, such as proteins, in a fluid sample by using the basic immunology concept of a specific binding between an antigen and its specific antibody. There are several different subtypes of ELISA,

but sandwich ELISA and competitive ELISA are the two most common ones (Gan & Patel, 2013).

During the sandwich technique, the well surface is prepared with a known quantity of bound antibody to capture the target antigen. After the non-specific binding sites have been blocked, the sample containing the target antigen is applied to the plate. To "sandwich" the antigen, a primary antibody specific to the target antigen is added. Secondary antibody-enzyme linked antibodies are then applied which bind to the primary antibody. The unbound enzymes are washed off with washing buffer. To measure the levels of the antigen, an enzyme substrate is added and is enzymatically converted to a color that can be quantified. The color intensity is directly proportional to the amount of antigen present in the sample (Figure 1) (Gan & Patel, 2013).

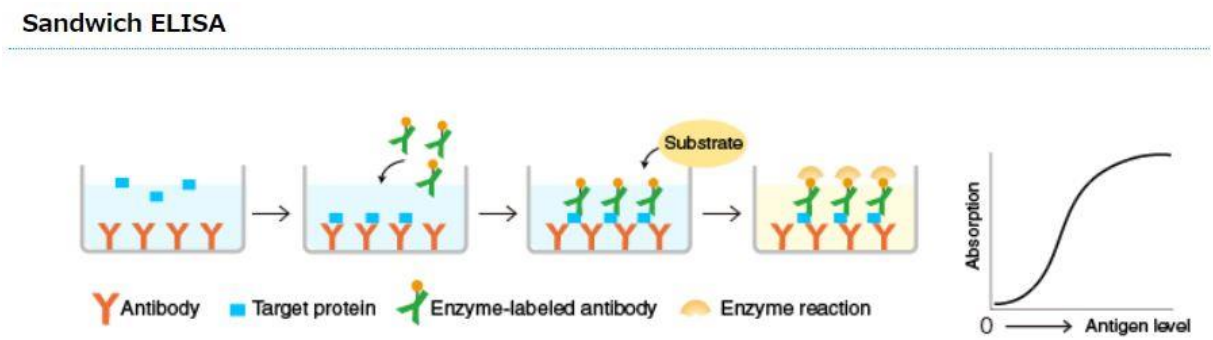


Figure 1: Sandwich ELISA technique ("The principle and method of ELISA," 2017).

The main event in competitive ELISA technique is the competitive reaction between a sample antigen and a competing labeled antigen that are added to the wells to compete in attaching to the antibody bound in the wells. Any remaining unbound antibody (labeled or sample) is washed off after a period of incubation. To measure the levels of the antigen, an enzyme substrate is added and is enzymatically converted to a color that can be quantified. The color intensity is reversible proportional to the amount of antigen present in the sample (Figure 2) (Gan & Patel, 2013).

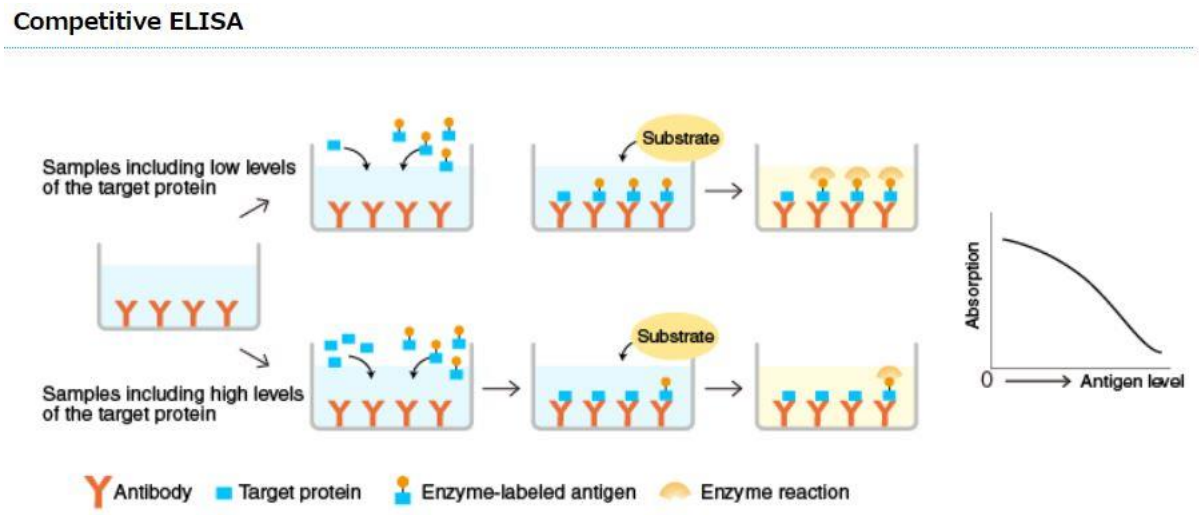


Figure 2: Competitive ELISA technique ("The principle and method of ELISA," 2017).

Commercial ELISA kits for specific antigens are available from several companies. The quality of an ELISA kit often depends on the antibodies used, and the quality of the kit can vary in terms of sensitivity, specificity, detection range and intra-assay variation. Sensitivity is the kits' ability to detect CA6. Specificity is the kits' ability of not reacting to substances that are not CA6. The detection range is the highest and lowest concentrations of CA6 the kits are able to detect. The intra-assay variation is the variation in results a data set collected from one experiment.

Although commercial ELISA kits for CA6 have been used in several studies (Esberg et al., 2019; Picco et al., 2019; Piekoszewska-Zietek et al., 2020), the basic quality parameters have not been published by independent research groups.

1.5. Research questions and hypotheses

The main objective of this study is to determine whether the commercially available ELISA kits are reliable for salivary CA6 concentration measuring. More specifically, we will examine the kits` sensitivity and specificity.

Our hypothesis is that the commercial ELISA kits "Company X" and "Company Y" are reliable for measuring CA6 concentration.

1.6. Utility value

The results of this study will determine the quality of the commercial ELISA kits for CA6. CA6 is a salivary enzyme whose physiological role is not fully understood but it is associated with caries increment and expression in several studies. This study will provide critical information to interpret and plan research on CA6.

2. Material and Methods

2.1. Approvals from Regional Ethics Committee and Norwegian Centre for Research Data

The study is a pilot for a study that has been given approval from the Regional Committees for Medical and Health Research Ethics, REK (approval number 2019/404) and the Norwegian Centre for Research Data, NSD (reference code 761592).

2.2. Ethics

The samples were collected after informed consent from two voluntary participants. Participation in this project was voluntary, the participants could withdraw their consent at any time, and they could then demand that the data would be deleted (if not already included in further analyses and/or published). The information collected did not have any diagnostic or therapeutic consequences for the participants. Obtaining biological information from saliva samples involved little or no risk or inconvenience to the participants. We determined only CA6 concentrations from the saliva samples. No analysis of human DNA was done in this project.

2.3. Study population

The samples were collected from the same two participants for every experiment.

2.4. Saliva collection

Paraffin-stimulated saliva was used for the measurements. A 1-gram piece of paraffin wax was given to the participants. They chewed the paraffin for 30 seconds and swallowed the secreted saliva. Thereafter, the subjects continued to chew on the paraffin and collected saliva for three minutes into a 50 ml Falcon tube.

The samples were centrifuged at 1000 x g at 4 C° for 15- 20 min. The supernatant was extracted and stored at -20°C until use.

2.5. Laboratory analysis

To measure CA6 concentration, we obtained ELISA kits from two different companies that we chose to name Company X» and "Company Y». One experiment was run with the "Company X" kit (competitive ELISA) and three experiments were run with the "Company Y" kits (sandwich ELISA) using a kit from different batch each time (Batch 1, 2 and 3). "Company Y" made changes for the kit in the 3rd batch. As positive controls in all kits, we used purified human CA6, which we received as a kind gift from Professor Seppo Parkkila´s group (University of Tampere, Finland).

The manufacturers` instructions were followed. The assay procedure briefly explained for the two kits:

1) Human CA6 (Carbonic Anhydrase VI) ELISA Kit, "Company X":
   Firstly, the plate was washed three times with washing buffer solution («Company X» washing buffer diluted 10:250 with distilled water). For standards, we used «Company X» standard diluted to 64 ng/ml, 32 ng/ml, 16 ng/ml, 8, ng/ml, 4 ng/ml, 2 ng/ml and 1 ng/ml in «Company X» sample/standard buffer. The «Company X» sample/standard buffer also served as the blank. The saliva samples were diluted 1:5, 1:20 and 1:100 in sample/standard dilution buffer. Standards, blanks and purified CA6 750 ug/ml (diluted 1:1000 in sample/standard dilution buffer), were added as duplicates into their respective wells. Subsequently, «Company X» Biotin-detection antibody diluted 1:100 in «Company X» Antibody dilution buffer was added to the wells and the plate was incubated for 45 min at 37°C. The wells were washed three times thoroughly with washing buffer solution. «Company X» HRP-Streptavidin Conjugate (SABC) diluted 1:100 in «Company X» SABC dilution buffer was added to the wells and the plate was incubated for 30 min at 37°C. The wells were thoroughly washed five times with washing buffer solution. «Company X» TMB substrate was added followed by a last incubation of 20 min at 37°C. The enzyme-substrate reaction was terminated by the addition of «Company X» stop solution, sulphuric acid solution, and the color change was immediately measured spectrophotometrically at a wavelength of 450 nm with a plate reader, Thermo Scientific MultiscanGO spectrophotometry.

2) "Company Y» CA6 ELISA kit:

For standards, we used «Company Y» standard solution diluted to 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62,5 pg/ml, 31,2 pg/ml and 15,6 pg/ml for batches 1 and two and 1,56 ng/ml, 3,12 ng/ml, 6,25 ng/ml, 12,5 ng/ml, 25 ng/ml, 50 ng/ml and 100 ng/ml for batch 3. The standard diluent also served as the blank. The saliva samples were diluted 1:100, 1:1000 and 1:3000 with distilled water. Distilled water was also used to dilute purified CA6 (750 ug/ml) 1:5000 and 1:50 000.

The saliva samples were added to the plate as triplicates and the standards, blank and purified CA6 were added as duplicates. Then, incubation for 60 min at 37°C was done before addition of Detection reagent A working solution («Company Y» Detection reagent A diluted 1:100 with «Company Y» Assay diluent A). The plate was incubated for 60 min at 37°C. The solution in the wells was aspirated and the plate was washed three times with wash solution («Company Y» wash solution concentrate diluted with distillated water 10:300). Detection reagent B working solution («Company Y» Detection reagent B diluted 1:100 with «Company Y» Assay diluent B) was added to the wells. After the plate had been incubated for 30 min at 37°C, the aspiration/wash process was repeated five more times. «Company Y» TMB solution was added to the wells for color development, following by an incubation 20 minutes at 37°C. «Company Y» Stop Solution was added, and the optical density (OD) measured immediately at 450 nm with a plate reader, Thermo Scientific MultiscanGO spectrophotometry.

Table 1: The specifications of the kits given in the instructions for use leaflets. CV= coefficient of variation. LLD=lower limit of detection. «COMPANY Y»=«Company Y»

	«Company X»	"Company Y" batch 1	«Company Y» batch 2	«Company Y» batch 3
Detection range	excellent	15,6-1000 pg/ml	15,6-1000 pg/ml	1,56-100 ng/ml
Intra-Assay CV	<8 %	<10%	<10%	<10%
Inter-Assay CV	<10 %	<12%	<12%	<12%
LLD		6,9 pg/ml	6,9 pg/ml	0,57 ng/ml

2.6. Statistical analysis

The program Curve expert version 1.40 (Hyams development) was used to make a standard curve based on known CA6 concentrations and corresponding ODs. Concentrations of the samples and controls were estimated from the standard curve.

3. Results

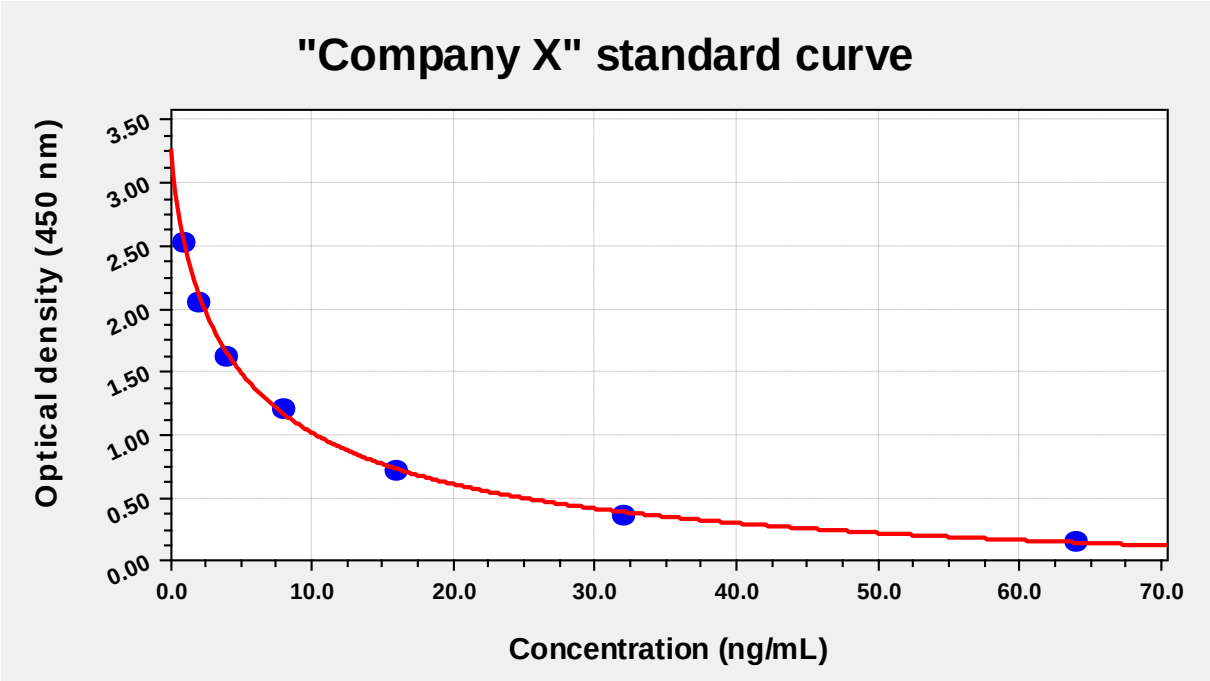


Figure 3: Standard curve for «Company X" CA6 ELISA Kit.

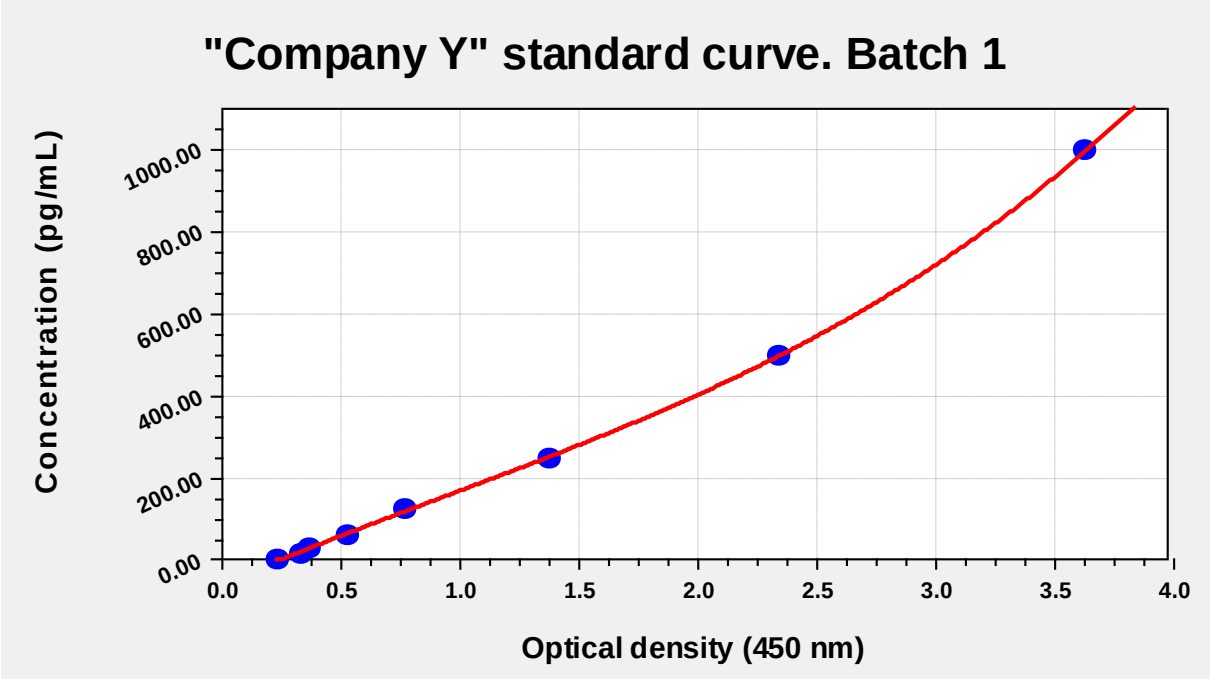


Figure 4: Standard curve for "Company Y» CA6 ELISA kit, Batch 1.

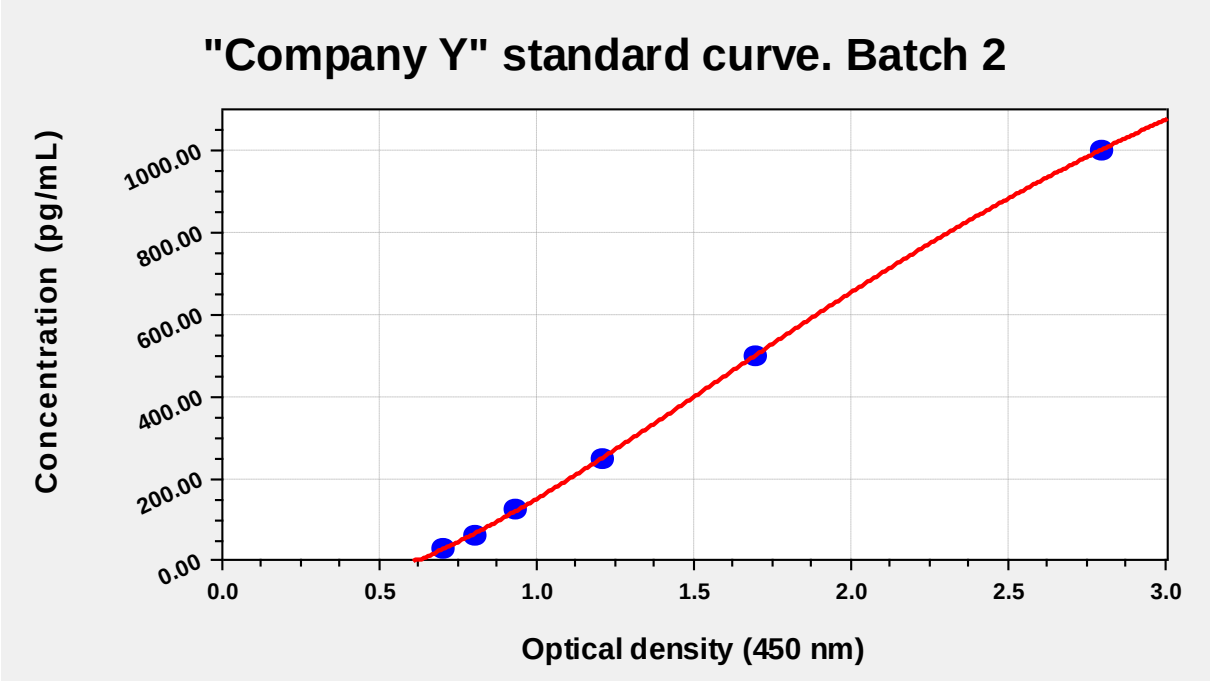
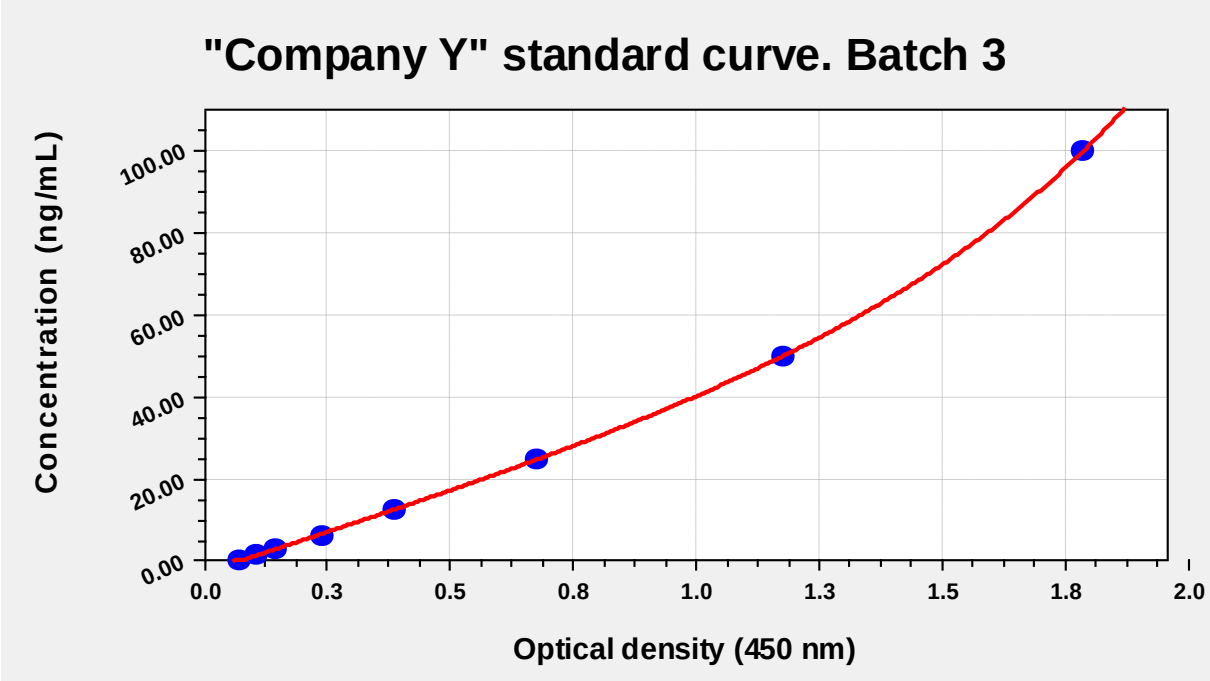


Figure 1: Standard curve for «Company Y» CA6 ELISA kit. Batch 2.



Figur 2: Standard curve for «Company Y»; CA6 ELISA kit, Batch 3

The shape of the «Company X» standard curve was exponential (Figure 3) and the shape of the standard curves from «Company Y» was linear (Figure 4, 5 and 6).

Table 2: The kits` detection of CA6 from purified CA6 ad saliva diluted 1:100.
** Mean concentrations of CA6 in saliva (diluted 1:100) for the two participants.*

ELISA kit	Purified CA6	Saliva*
«Company X» ELISA kit	0	0,32 mg/L
«Company Y» ELISA kit batch 1	0	0
«Company Y» ELISA kit batch 2	0	0
«Company Y» ELISA kit batch 3	0	11,2 mg/L

None of the kits detected the purified CA6 (Table 2). Only the «Company X» kit and the batch 3 of «Company Y» Kits gave a positive reaction in saliva samples (Table 2).

4. Discussion

Our results show that none of the kits detected CA6. Only the «Company X» kit and the batch 3 of the «Company Y» kits gave positive reactions from the saliva samples (Table 2). But this must be either unspecific reaction or specific reaction but to other proteins than CA6. Since the «Company X» kit and the batch 3 of the «Company Y» kits did not detect the purified CA6, but reaction from the saliva samples, a possible explanation could be that the kits have an unspecific binding to another protein in the samples. In other words, the kits lack specificity.

The fact that the «Company X» standard curve is exponential, gives a limited optimal detection range for CA6 measuring (Figure 3). For the «Company X» kit, the CA6 concentrations should not exceed the optimal detection range as the values in this part of the curve will not rise heavily by small changes in OD. Small pipetting inaccuracies could cause major differences in the determined CA6 concentration values the further away the ODs goes from the optimal range. Even though the samples were diluted to fit in the optimal range, it was not possible to have the samples of both participants fitting into the optimal range at the same dilution. There is wide variation in CA6 concentrations both between individuals and intra-individually according to sleeping and eating patterns (Kivela, Parkkila, Parkkila, Leinonen, et al., 1999). This variation will cause many of the samples to be outside the optimal area of the curve. The «Company X» standard curve is far from optimal when measuring CA6 concentration of saliva samples. The standard curve from the «Company Y» kit had a linear shape (Figure 3,4 and 5) avoiding this problem.

There is no guarantee for absence of human flaws as the laboratory work was done by humans. The lab work required precise pipetting with small volumes, increasing the chance for inaccuracies. However, we repeated the experiments for «Company X» and «Company Y» kits batch 1 and 2 to check for mistakes in the procedure, equipment and reagents. To reduce the risk of bias between the experiments, the same examiner performed most of the experiments (exception is Cloud-Clone. Corp batch 3). The experiments were designed as similar as possible (e.g. all four of the experiments had a 1:100 saliva dilution). Moreover, the standard curves from the experiments are similar to the ones given in the company protocols, which indicates that the laboratory procedure was conducted without major errors.

The most likely explanation for the results obtained in this study could be that the kits lack the ability to detect CA6. However, it is difficult to assess the kits in detail because the companies do not provide specific information on the content in the reagents. This makes troubleshooting difficult. It should also be mentioned that it might be unprofitable for companies to invest economically in developing commercial ELISA kits for CA6, since the demand for them is sparse. Nevertheless, the fact that the commercial kits have been used in a limited amount of experiments rises the probability of defects not yet being discovered.

A strength of the study is that the positive control, purified CA6, was independent and not linked to any of the companies. Instead, we received the purified CA6 as a kind gift from Seppo Parkkila´s group who are the leading research group in the field of CA6 since 1990 (Parkkila et al., 1990).

We collected saliva samples from the same two participants in all the experiments, and both of the samples were present in every experiment. The collection procedure was identical for each sampling. However, a possible limitation of the study is that the same participant donated different samples (three samples at three different days), which were used in the experiments. One can think that the fact that the samples were collected at different days could cause different protein compositions in the samples. This gives unnecessary inter-experiment variations that preferably should have been avoided. However, this will not affect the outcome of this study since comparison of the kits to one another was not the purpose of the study.

The findings of this study indicate that one should be vary of studies where commercial uncertified kits have been used. It might be that the results of these studies are based on unspecific binding. This is to our knowledge the only study examining the quality of commercial CA6 ELISA kits. The results obtained in this study indicate that quality assessment of commercial ELISA kits is necessary, at least for CA6 kits if not to other kits as well.

5. References

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