



An IgY-based immunoassay to evaluate the biomarker potential of the *Tannerella forsythia* virulence factor karilysin in human saliva

Peter Durand Skottrup^{a,b,*}, Rodrigo López^c, Mirosław Ksiazek^{d,g}, Peter Højrup^e, Vibeke Baelum^f, Jan Potempa^{d,g}, Jakub Zbigniew Kaczmarek^{e,h}

^a Department of Clinical Biochemistry, Copenhagen University Hospital, Hvidovre DK-2650, Hvidovre, Denmark

^b Novo Nordisk A/S, Global Research Technologies, Research Bioanalysis, Novo Nordisk Park, DK-2760 Måløv, Denmark

^c Section of Periodontology, HEALTH, Aarhus University, DK-8000 Aarhus, Denmark

^d Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

^e Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense M, Denmark

^f Section for Oral Epidemiology and Public Health, HEALTH, Aarhus University, DK-8000 Aarhus, Denmark

^g Department of Oral Immunology and Infectious Diseases, University of Louisville, School of Dentistry, Louisville, KY, USA

^h Research and Development Department, Sanovo Biotech A/S, DK-5000 Odense, Denmark

ARTICLE INFO

Keywords:

Periodontitis
Inflammation
Virulence factor
Biomarker
Saliva
Karilysin
IgY

ABSTRACT

Tannerella forsythia is a gram-negative anaerobic bacterium that is associated with the development of destructive periodontal disease. *T. forsythia* secretes the metalloprotease-like enzyme karilysin. Using *in vitro* systems karilysin has been shown to modulate the host immune response by degradation of complement system proteins and by inactivation of the antimicrobial peptide LL-37 by proteolytic cleavage. This makes karilysin a highly interesting virulence factor to study in the framework of drug development and diagnostics. However, to date the presence of karilysin in clinical samples has not been demonstrated due to the lack of specific probes.

In the present work, a high titer and stable affinity-purified avian IgY antibody against karilysin was developed. By surface plasmon resonance imaging the IgY affinity was found to be in the low nanomolar range. The antibody could be used to detect karilysin in saliva samples by immuno-blotting and was specific when tested towards human MMP-3. Furthermore, an avian IgY-based immunoassay was developed, which demonstrated low intra- and interday assay variability (CV's below 10%).

Application of the immunoassay on a well-characterized set of saliva samples from adolescents with or without signs of periodontitis showed that it was possible to detect karilysin in saliva. A significant difference in karilysin concentration was found between saliva from participants with signs of periodontitis and saliva from healthy controls ($p = .0024$). The median of karilysin levels among periodontitis cases was 957 pg/ml (IQR, 499–2132 pg/ml) and the median for controls was 569 pg/ml (IQR, 210–1343 pg/ml).

Collectively our data confirm the presence of karilysin in clinical samples. The described IgY-based immunoassay may prove useful as part of protein-based biomarker screenings in the clinic or in point-of care settings.

1. Introduction

Periodontitis is a chronic, destructive inflammatory disease possibly resulting from the interaction between a dysbiotic microbial community in the oral cavity and the host response. Periodontopathogens release virulence factors, which contribute to inflammation and loss of

tissue attachment to the root of teeth. This in turn can lead to permanent destruction of supporting tissue and ultimately tooth loss (Imamura, 2003). The disease is widespread with more than 40% of adults in the United States experiencing periodontitis (Eke et al., 2015). Recently studies have also suggested that severe forms of periodontitis contribute to development of systemic diseases, such as rheumatoid

Abbreviations: ELISA, Enzyme-linked immunosorbent assay; *T. forsythia*, *Tannerella forsythia*; TNF α , Tumour necrosis factor alpha; PBS, Phosphate buffered saline; IgY, Immunoglobulin Y; PVDF, poly(vinylidene difluoride); TCA, trichloroacetic acid; LOQ, Limit of quantification; DTT, dithiothreitol; SPR, Surface plasmon resonance

* Corresponding author at: Novo Nordisk A/S, Global Research Technologies, Research Bioanalysis, Novo Nordisk Park, DK-2760 Måløv, Denmark.

E-mail address: peter.skottrup@gmail.com (P.D. Skottrup).

<https://doi.org/10.1016/j.jim.2019.03.003>

Received 21 November 2018; Received in revised form 21 February 2019; Accepted 13 March 2019

Available online 14 March 2019

0022-1759/ © 2019 Elsevier B.V. All rights reserved.

arthritis, diabetes and stroke.

The ‘red complex’ is a term used for the three key periodontopathogens (*Treponema denticola*, *Porphyromonas gingivalis* and *Tannerella forsythia*), which are suspected to be involved in disease development (Socransky et al., 1998). These pathogens all secrete proteases that degrade oral cavity proteins and the pathogens scavenge the released amino acids for growth. This proteolytic activity is believed to contribute to the periodontitis symptoms by destruction of soft tissue and bone. (Jiao et al., 2014; Sochalska and Potempa, 2017).

Karilysin is a metalloproteinase-like enzyme secreted from the periodontopathogen *T. forsythia*. Karilysin is a 472-residue protein, which has been recombinantly expressed and mechanistic studies have revealed that the enzyme matures through sequential autolysis, by first generating a fully active 48 kDa variant, followed by formation of the catalytic domain (named Kly18) (Karim et al., 2010). Functional analysis has identified karilysin as an inactivator of the antimicrobial peptide LL-37 by proteolytic cleavage (Koziel et al., 2010). Furthermore, evidence suggests that karilysin-expressing *T. forsythia* isolates inhibit all pathways of the complement system by karilysin-mediated degradation of complement system proteins (mannose-binding lectin, ficolin-2, ficolin-3, C4 and C5) (Jusko et al., 2012). Another piece of evidence is the newly proposed karilysin-mediated cleavage of the membrane form of TNF α (Bryzek et al., 2014). This effect releases TNF α leading to an inflammatory response by recruitment of immune cells.

The evidence above suggests that karilysin contributes to evasion of the human immune response and that it could be considered a potential therapeutic target. To pursue this we recently identified a tetrameric peptide competitive inhibitor of karilysin that could form the basis for a peptidomimetic drug development approach. (Skottrup et al., 2012; Guevara et al., 2013). However, the presence of karilysin in clinical samples has not been investigated due to the lack of specific antibodies.

In this study, we developed and characterized an affinity-purified avian IgY antibody and qualified a competitive immunoassay for detection of karilysin in saliva. Using the IgY-based immunoassay we find that karilysin in saliva is positively correlated with signs of periodontitis in adolescent saliva.

2. Materials and methods

2.1. Chicken immunization and IgY purification

All animal experimental protocols complied with current ethical standards for the use of laboratory animals according to European regulations. As the immunizations were performed at a contract research organisation in Sweden, the ethical approval comes from The National Committee for the Protection of Animals Used for Scientific Purposes, at the Swedish Agricultural department. The hens were housed in approved facilities for laboratory animals according to European regulations. Three 20-week-old white leghorn hens were kept in individual cages with food and water *ad libitum*. Hens were immunized intramuscularly with recombinant purified Kly18 (Karim et al., 2010) with Freund's adjuvant (Thermo Scientific, Massachusetts, USA) at different sites of breast muscle. For the first immunization each hen received 100 μ g of solubilized antigen mixed in 1:1 ratio with Freund's complete adjuvant. Three booster immunizations were performed using Freund's incomplete adjuvant with four weeks intervals. After two weeks from the last boost immunization, the eggs were collected, marked and subsequently stored at 4 °C until they further use for IgY extraction.

Before the purification of the whole egg yolk antibody fraction the egg yolks were semi-manually separated from the whites and prior to the egg yolk sack puncture, they were thoroughly washed with deionized water in order to remove any remaining egg white proteins. The yolks were separated from the membranes and processed further.

Whole IgY egg yolk fraction was separated by Sanovo Biotech's

proprietary technology in a one-step chromatographic purification process. The antibodies were then eluted from the system in a non-denatured form in the absence of any toxic chemicals. The final antibody fractions were formulated in PBS buffer pH = 7.4 supplied with 0.09% sodium azide and the antibody concentration was determined to be 14.3 mg/mL using the Bradford assay (Sigma B6916).

The Kly-18 IgY fraction was further affinity purified on immobilized Kly18. The affinity resin was prepared by conjugating 5 mg of Kly18 (in PBS pH = 7.4) to 450 μ g of Pierce NHS-Activated Agarose according to the manufacturer's instructions (Pierce product no 26196). The Kly18-functionalized resin was manually packed into an XK 16/20 column (GE Healthcare) and washed extensively with PBS pH = 7.4. Affinity purification was done against the whole IgY fraction in 3 subsequent runs on a fully automated ÄKTA pure system (GE Healthcare). The specific antibody fraction was eluted using isocratic pH elution with 100 mM glycine-HCL buffer pH = 2.8 and eluted IgY was immediately neutralized with 1 M Tris-HCL pH = 8.5. The obtained IgY fractions were pooled and buffer exchanged to PBS pH = 7.4 supplemented with 0.02% sodium azide on a HiPrep 26/10 desalting column (GE Healthcare). The final IgY was concentrated to 1 mg/mL using Sartorius Vivaflow 20 concentrators (MWCO 30 kDa) and the concentration was verified using Bradford analysis. As a control, anti-D-dimer IgY was used in the experiments (lot: 287–16-DIM-1-1, Sanovo Biotech). The control IgY was raised against D-dimer and specific IgY's was affinity purified on D-dimer-functionalized resin according to the same protocol as above.

The purity of both, whole IgY and affinity purified IgY was tested by SDS-PAGE on Run Blue (NXG41212K) 4–12% Bis-Tris polyacrylamide gel (Expedeon). Separation was performed on Easy Power 500 (Invitrogen) in a non-reduced environment for 45 min at 200 V, 110 mA. The gel was visualized with Coomassie Simply Blue Safe Stain (Invitrogen LC6060) and developed according to the manufacturer's instructions. Gels were scanned and Genetools software (Syngene) was used to estimate IgY purity by image analysis.

2.2. Dot blot analysis

Purified Kly18 was blotted to methanol-activated poly(vinylidene difluoride) (PVDF) membrane (Expedeon, run blue) and allowed to dry. Membranes were blocked for 30 min in 5% (w/v) skimmed milk in 20 mM Tris-HCL and 150 mM NaCl at pH 7.4 (TBS), followed by 5 times washing in washing TBS with 0.1% Tween20 (TBS-T). Anti-Kly18 IgY was diluted to 1/2000 in TBS-T and incubated with the membrane for 30 min followed by washing as above. Secondary anti-IgY antibody (Rabbit anti-chicken-HRP A9046, Sigma-Aldrich) was diluted to 1/30000 in TBS-T and incubated for 30 min and the membrane was washed as above. The blot was developed by enhanced chemiluminescence (ECL, GE Healthcare).

2.3. Polyacrylamide Gel Electrophoresis (PAGE) and Western blotting

Proteins were separated by SDS-PAGE in polyacrylamide gels (precast gels, 10% Expedeon, run blue). Prior to electrophoresis, samples were boiled for 5 min in the presence of 30 mM dithiothreitol (DTT) and 1% SDS (sample buffer). For Western blotting, proteins were transferred to PVDF membrane as described before (Semi-dry blotter, Trans-Blot SD Semi-dry transfer cell) (Kyhse-Andersen, 1984). The membrane was blocked with 3% (w/v) skimmed milk in TBS and was developed as above. Active human MMP-3 catalytic domain (Sigma-Aldrich) was included as a specificity control. MagicMark™ XP Western Protein Standard was included to estimate molecular weight. Saliva samples were precipitated using trichloroacetic acid (TCA) as described (Jiang et al., 2004). The dried samples were redissolved in sample buffer.

2.4. Indirect ELISA for titer determination

Purified Kly18 was coated in Nunc Maxisorp plates at 5 µg/ml for one hour at 37 degrees. Wells were emptied and blocked for one hour with 200 µl 1% skimmed milk in PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.4). Wells were washed with PBS containing 0.05% Tween20 (PBS-T) four times. Next, wells were incubated with affinity-purified anti-kly18 IgY diluted in PBS-T for one hour. Wells were washed as above, and anti-IgY antibody was added (Rabbit anti-chicken IgY-HRP, Sigma-Aldrich, A9046, diluted 1/1000 in in PBS-T) for one hour. The ELISA was developed using 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma-Aldrich) as the substrate with acid stop (read at 450 nm).

2.5. Competitive ELISA development

The competitive ELISA was performed essentially as described earlier (Skottrup et al., 2011). Briefly, Nunc Maxisorp plates were coated with 5 µg/ml Kly18 and blocked with 1% (w/v) skimmed milk powder/PBS as described above. A titration of Kly18 IgY in PBS-T (using the indirect ELISA setup described above) revealed the titer that gave half-maximum response and this Kly18-IgY dilution was used for the inhibition assay (titer 1/16000). The Kly18-IgY titer concentration was incubated with decreasing concentrations of Kly18 in Kly18-coated/blocked wells (5 µg/ml Kly18) for one hour. Wells were washed with PBS-T and further incubated with rabbit anti-chicken IgY-HRP (diluted 1/1000 in PBS-T) for one hour. After the final PBS-T wash, the absorbance values were measured at 450 nm after 30-min incubation at 22 °C. OD₄₅₀ values at each Kly18 concentration (R) were divided by the OD₄₅₀ value at zero Kly18 (R₀). These normalized values (R/R₀ × 100%), were plotted against the Kly18 concentration to make the competition curve and a curve was fitted using a four-parameter equation. The assay was performed in duplicate measurements. The assay was performed 5 times on the same day and on three individual days to generate data on the assay intra-day and inter-day performance. Limit of quantification (LOQ) was calculated as follows;

$$LOQ = Mean - 10 \times SD$$

where *Mean* represents the mean of seventeen blank samples and *SD* is standard deviation from the seventeen blank samples.

2.6. Surface plasmon resonance imaging (SPR-imaging)

Kly18 was immobilized on the SensEye G Easy2spot sensor as two fold dilutions in sodium acetate buffer pH 4.5 starting from 512 nm to 0.25 nm. Kly18 spots were printed on the sensor by the use of CFM (Wasatch Microfluidics, Salt Lake City, UT, USA). Experiments were performed on an IBIS MX96 (Ibis Technologies BV., Enschede, The Netherlands). TBS 1 × buffer with 0.05% Tween was used as running buffer. Prior to experiment start the printed sensor was quenched with 1 M ethanolamine pH 8.5 for 7 min followed by flushing with running buffer. The experimental setup included IgY injections ranging from 512 nm to 0.25 nm prepared as two fold dilutions. The antibody dilutions were prepared in the running buffer in order to decrease the refractive index differences. Anti Kly18 polyclonal antibodies were applied on the printed sensor surface followed by a regeneration step with glycine-HCL pH 3 for 1 min after each injection. After each fourth sample injection the sensor was washed with running buffer in order to record signals with no associations of the ligand. The complexes were allowed to associate for 3 min followed by 6 min dissociation in the running buffer. The data was recorded using DAX software (IBIS Technologies) and processed using SPRint (IBIS Technologies) in order to obtain quantitative measures of the antibody-antigen interactions. A double referencing step was included as described previously (Myszka, 1999). The sensorgrams were analyzed in Scrubber2 software where the recorded association and dissociation curves were fitted globally into a 1:1 binding model. The equilibrium dissociation constant (KD)

for all analyzed antibody concentrations interacting with each captured Kly18 spot was determined by the ratio of the kinetic rate constants, $KD = k_d/k_a$.

2.7. Analysis of saliva samples from adolescents with signs of periodontitis and adolescent control samples

The study group originates from a case-control study of Chilean adolescents with clinical signs of periodontitis and consists of 160 adolescents; 87 cases with periodontitis and 73 healthy controls. The reader is referred to reference (Lopez et al., 2001) for detailed description of the sampling strategy and demographic profile of the underlying study identifying cases and controls and to (Lopez et al., 2009) for description of the clinical features of this subsample of subjects participating in the case-control study. The study protocol was approved by the local Committee of Ethics of the University of Chile and participation was based on written informed consent and parental approval.

Saliva was collected as previously described (Lopez et al., 2001; Lopez et al., 2009; Lopez et al., 2011a) and initially stored at -20 °C in Chile. Samples were then transferred to Aarhus University, Denmark for -80 °C storage and in 2015, the samples were moved to the Department of Clinical Biochemistry, Copenhagen University Hospital at Hvidovre and stored at -80 °C. Extensive studies using the saliva samples have been performed and we recently demonstrated that the biomarker suPAR could be detected in the samples and used as a marker for signs of adolescent periodontitis (Skottrup et al., 2018). Furthermore, repeated freeze/thaw cycles and re-analysis confirmed that the sample integrity was preserved.

The 160 saliva samples were analyzed in duplicate in the competitive ELISA described above. Prior to analysis saliva samples were centrifuged at 5000 × g for 10 min at 4 °C. Dilution recovery in assay dilution buffer (PBS-T) was validated using saliva from seven individual samples. Samples were diluted 1:2 in assay dilution buffer prior to analysis.

2.8. Kly18 activity assay

The Kly18 protease activity was monitored essentially as described (Karim et al., 2010; Skottrup et al., 2012). Briefly, assays were performed at 37 °C using 500 nM of Kly18 in assay buffer (100 mM Tris-HCl, 5 mM CaCl₂, pH 8.0). Kly18 was mixed with varying concentrations of Kly18 IgY followed by 30 min incubation at 22 °C. The substrate (FITC-casein, Sigma-Aldrich 25 µg/ml) was added and fluorescence (Ex. 490 nm, Em. 525 nm) was monitored at 37 °C for 60 min with measurements every ten minutes.

2.9. Statistical analysis

The data were analyzed using GraphPad PRISM® (version 7.03, GraphPad Software, Inc. La Jolla, CA, USA). Normality test demonstrated that the case/control were not normal distributed. Consequently, Mann-Whitney tests were performed. The data are presented as median and interquartile ranges (IQR).

3. Results

3.1. IgY production and purification

The karilysin catalytic domain (Kly18) was recombinantly expressed as described previously (Karim et al., 2010) with purity sufficient for immunization (data not shown). The purified Kly18 was used to immunize three white leghorn hens with four-week intervals in four consecutive antigen injections. The immunization scheme in terms of dosing and immunization frequency is seen in Table 1. The egg yolk was harvested and the IgY fraction was enriched by a one-step purification

Table 1

Immunization scheme of three white leghorn hens. Kly18 was used as the antigen in all immunizations.

Immunization nr	Kly18 antigen quantity (µg)	Adjuvant used	Time point
First	100	Freunds complete	Week 1
First boost	100	Freunds incomplete	Week 4
Second boost	100	Freunds incomplete	Week 8
Third boost	100	Freunds incomplete	Week 12

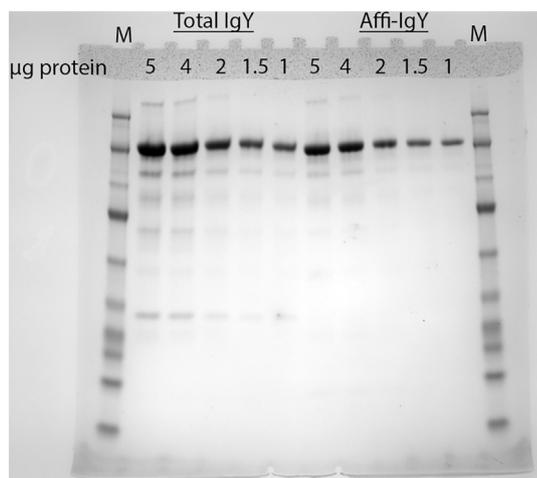


Fig. 1. SDS-PAGE analysis. IgY was purified by Kly18-affinity chromatography. Shown are total IgY prior to purification and after affinity purification. GeneTools was used to estimate IgY purity. Total IgY was estimated at 47% purity, whereas Kly18-affinity purified IgY was 91% pure. MwM is short for molecular weight marker.

strategy. The total IgY fraction was further purified by Kly18-affinity purification, leading to an increase in IgY purity from 47% to 91% (Fig. 1). Immunoreactivity of the affinity purified Kly18 IgY was confirmed by indirect ELISA and a specific binding profile was found, with a 125× increase in ELISA-titer. Immunoreactivity of the affinity purified Kly18 IgY was evaluated by indirect ELISA and a specific binding profile was found, Fig. 2A.

3.2. Kly18 IgY characterization

SPR-imaging measurements of the Kly18-IgY complexes was performed in a multiplex format that allowed the Kly18 spots printed on the sensor to react with antibody injections in real-time. The data for each Kly18-spot was recorded separately in real time, which greatly increased the throughput and decreased the total experiment time. After scaling the spots used for estimating the on and off-rates of the IgY-Kly18 complexes were narrowed down to those exemplifying best the 1:1 binding model curve fits. The 265 nm Kly18 spot reacting with 512 nm, 265 nm and 128 nm IgY injections was used for further analysis. The fitted data was used to estimate the rate constants and the overall affinity of the polyclonal antibody preparation. All three antibody injections on the 265 nm immobilized Kly18 spot formed relatively stable complexes with averaged K_D values in the low nanomolar range as indicated in Fig. 2B. The estimated affinity in this range is acceptable for a polyclonal antibody preparation of this type (Conroy et al., 2012). The low decaying shape of the curve indicates that the complexes are relatively stable over time (Fig. 2B).

The affinity-purified Kly18-IgY was characterized by dot-blot analysis and optimal dilutions for Kly18-IgY and secondary anti IgY-HRP antibody were identified (data not shown). The dot-blot data in Fig. 3A demonstrates that Kly18 can be detected by the Kly18 IgY but not a control IgY raised against human D-dimer. Furthermore, by adding Kly18 in solution for competition the binding signal disappears, thereby

confirming the specific interaction of Kly18 IgY. Lastly, the catalytic domain from human MMP3, the protein with the highest sequence similarity to Kly18, could not be detected by the Kly18 IgY. By Western blotting it was furthermore possible to verify that the Kly18 IgY could detect the SDS-denatured form of Kly18 and the absence of signal in the anti-D-dimer panel confirms the Kly18-specific binding of anti-Kly18 IgY (Fig. 3B). Detection of native karilysin was confirmed in saliva from five random subjects with signs of periodontitis and four random controls, Fig. 3C.

The Kly18 IgY was furthermore characterized in a Kly18 functional assay, where the Kly18 proteolytic activity was monitored with FITC-casein as the substrate. By pre-incubation of Kly18 with increasing concentrations of Kly18 IgY (up to 2 µM), no inhibition of the Kly18 proteolytic activity was observed (data not shown).

3.3. Development of competitive ELISA for karilysin detection

In order to quantify karilysin in saliva a competitive IgY-based ELISA was developed. Assay conditions were optimized and a calibration curve within 0–24,000 pg/ml was constructed, Fig. 4. The assay demonstrated good intra-day and inter-day stability, with coefficient of variation percentages below 10% for the entire measuring range, Table 2. The assay limit of quantification (LOQ) was 161 pg/ml.

The competitive ELISA was used to quantify karilysin in saliva from adolescents with periodontitis and control samples. By Mann-Whitney test a significant difference in karilysin concentration was found between controls and periodontitis cases ($p = .0024$). The median of periodontitis cases were 957 pg/ml (IQR, 499–2132 pg/ml), whereas the median for controls was 569 pg/ml (IQR, 210–1343 pg/ml) (Fig. 5). Twelve periodontitis cases and thirteen controls had undetectable levels of karilysin.

4. Discussion

Understanding the composition of the saliva proteome is important in disease biomarker discovery. Saliva is an extremely complex matrix with multiple proteins present (Schulz et al., 2013). Establishing the tools in terms of antibodies and assays is important to enable in depth characterization of this matrix in particular when it comes to pathogen-derived virulence factors as they may represent potential molecules of diagnostic relevance.

Karilysin from *T. forsythia* has been proposed as a virulence factor by degrading several components of the human immune system (IL-37, TNF- α and complement system factors) (Koziel et al., 2010; Jusko et al., 2012; Bryzek et al., 2014). This *in vitro* evidence underlines the importance of karilysin in the pathogenesis of *T. forsythia*. However, limited *in vivo* evidence for the presence of karilysin in human samples exists. In a recent study (Siddiqi et al., 2016), quantitative real-time PCR assays were developed for detection of *S. aureus*, *P. gingivalis* and *T. forsythia* in tongue samples from edentulous patients. The *T. forsythia* primers were based on the karilysin gene sequence, but the authors were not able to detect any of the bacterial species in 26 patients.

The saliva samples used in the present study have earlier been screened for the presence of 18 bacterial species (Lopez et al., 2011b) at the DNA-level. Molecular hybridization techniques demonstrated that all eighteen bacterial species were detectable, including the members of

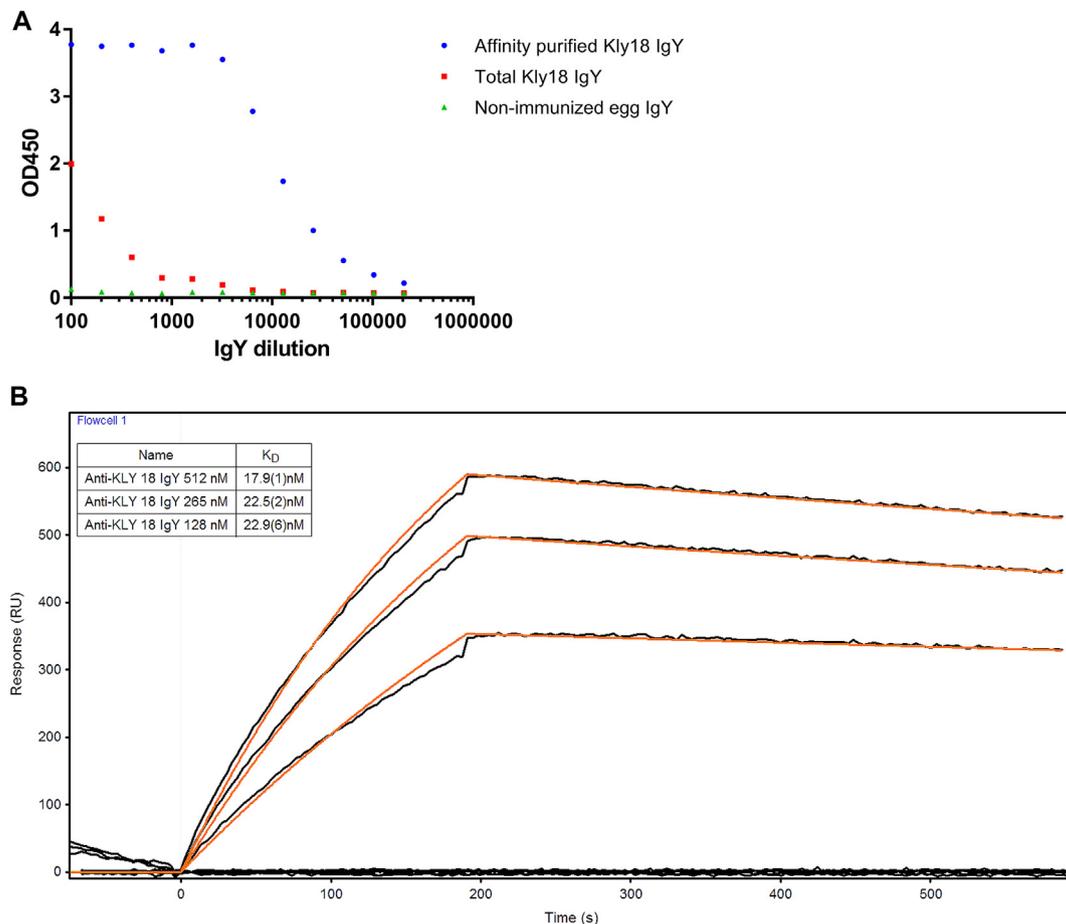


Fig. 2. A) Direct ELISA Kly18-IgY titer determination. Direct ELISA was used to estimate the increase in titer following affinity purification. ELISA-titer of total IgY was estimated at 1/100 (10 µg/ml) and ELISA-titer for affinity purified IgY was 12,800 (0.08 µg/ml). B) Single 256 nm Kly18 spot reacting with 512 nm (top curve), 256 nm (middle curve) and 128 nm (bottom curve) anti-Kly18 IgY antibody injections in real-time. Association and dissociation curves of the antigen-antibody complexes are indicated in black and the curve corresponding fits are shown in orange. The rate constants were determined using Scrubber 2 software after the association and dissociation curves were fitted into a 1:1 interaction model. Single KD values are presented for each respective curve fit.

the red-complex, *T. forsythia* and *P. gingivalis* (Lopez et al., 2011b). Using the saliva sample panel we recently demonstrated that periodontitis-derived inflammation could be detected in saliva from the adolescents with symptoms of periodontitis using the biomarker suPAR (Skottrup et al., 2018).

To date knowledge of the presence of karilysin protein in human samples has not been verified, due to the lack of specific assays. Consequently we used IgY-technology to develop a high affinity and stable probe capable of discriminating between Kly18 and the protein with the highest sequence identity namely human MMP-3. The high-performing IgY was used to develop a competitive immunoassay capable of detecting karilysin in saliva. The assay was reproducible and specific and had a sensitivity that is on par with other competitive ELISAs for protein biomarkers in saliva (Schwartz and Granger, 2004; Dag et al., 2010).

Given that, the adolescent saliva samples were proven positive for *T. forsythia* (Lopez et al., 2011b), they were suitable for the objective of this study that was to explore whether karilysin could be detected in saliva using our novel IgY-based ELISA. Our data demonstrated that karilysin was present in saliva samples in both periodontitis cases and in the control samples. However, we did observe a significant difference in the karilysin concentration between periodontitis cases and controls. This result is interesting and is, to the best of our knowledge, the first finding of a virulence factor from a putative periodontopathogen that could be associated with saliva with increased periodontitis-derived inflammatory activity.

Identifying protein-based biomarkers in the clinic or in point-of-care settings is an interesting field of research. If successful, these biomarkers could aid diagnostics by predicting, diagnosing and determining the progressive phases of episodic periodontitis. Furthermore, such tests could be used to evaluate treatments and medications. Along these thoughts, a recent study evaluated endogenous matrix metalloproteinase-8 (MMP8) and interleukin-1β from gingival crevicular fluid, mouthrinse and saliva. The findings were that MMP8 can be used alone or alternatively together with interleukin-1β and *P. gingivalis* presence to calculate cumulative risk scores at the subject level as a successful diagnostic tool. The authors suggest that this method could be used as a rapid screening tool in large-scale public health surveys, where thorough periodontal examinations may not be rational (Sorsa et al., 2016). Karilysin could be evaluated in similar studies perhaps in combination with other periodontopathogen virulence factors such as RgpB from *P. gingivalis*. Indeed, we recently developed as nanobody specific for the *P. gingivalis* virulence factor RgpB, which could prove useful for this purpose (Skottrup et al., 2011).

It should be noted here that more studies are needed in order for karilysin to become a documented biomarker. Clearly, some non-periodontitis participants have *T. Forsythia* in the oral cavity, which is also the case for the presently used well characterized subject population with early signs of periodontitis (Lopez et al., 2011b). Therefore, diagnostic and validation studies must be performed to establish reference intervals in the adult population and other subpopulations such as children and the elderly, so that cut-off values can be validated.

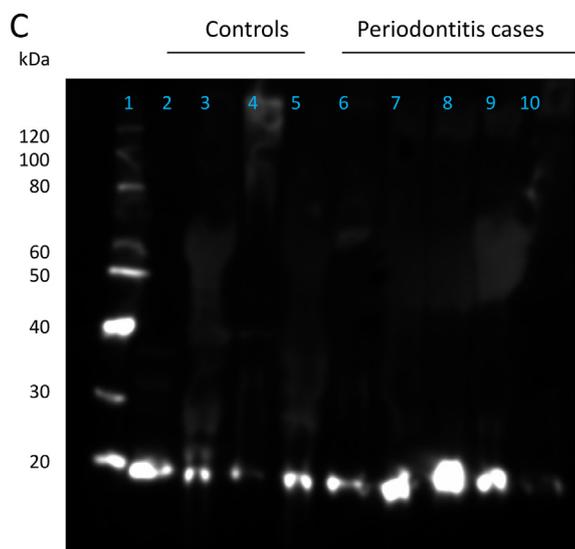
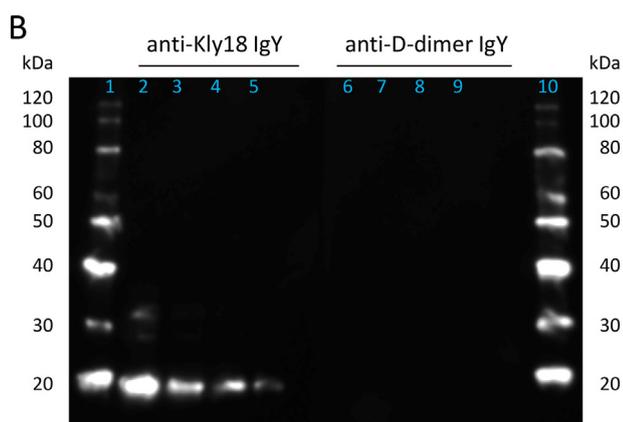
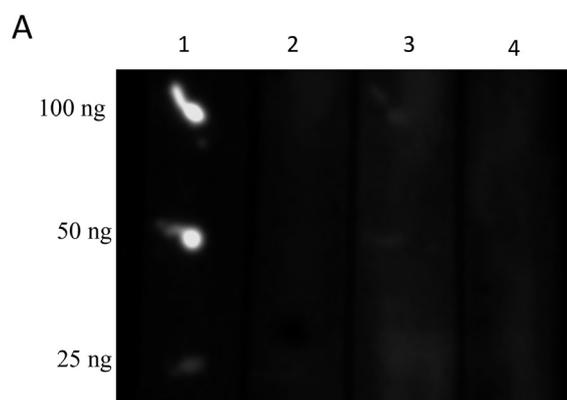


Fig. 3. A) Dot blot analysis of anti-Kly18 specificity. Lane 1: Kly18 blotted and analyzed with anti-Kly18 IgY, Lane 2: Kly18 blotted and analyzed with control IgY (D-Dimer), Lane 3: Kly18 blotted and analyzed with anti-Kly18 IgY + 10 μ g Kly18, Lane 4: Human MMP3 (catalytic domain) blotted and analyzed with anti-Kly18 IgY. B) Western blot analysis demonstrates that the Kly18 IgY binds to SDS-denatured Kly18. Lane 1 + 10 MagicMark™ XP Western Protein Standard, lane 2 + 6 (0.1 μ g Kly18), lane 3 + 7 (0.01 μ g Kly18), lane 4 + 8 (0.001 μ g Kly18) and lane 5 + 9 (0.0001 μ g Kly18). In lanes 2–5 anti-Kly18 IgY was used for detection and in lanes 6–9 anti-D-dimer IgY was used for detection. The absence of signal in the anti-D-dimer panel confirms the Kly18-specific binding of anti-Kly18 IgY. C) Karilysin can be detected in saliva samples from cases and saliva from subjects without signs of periodontitis by western blot analysis. Lane 1, MagicMark™ XP Western Protein Standard, Lane 2–5 (periodontitis cases), lane 6–10 (subjects without signs of periodontitis).

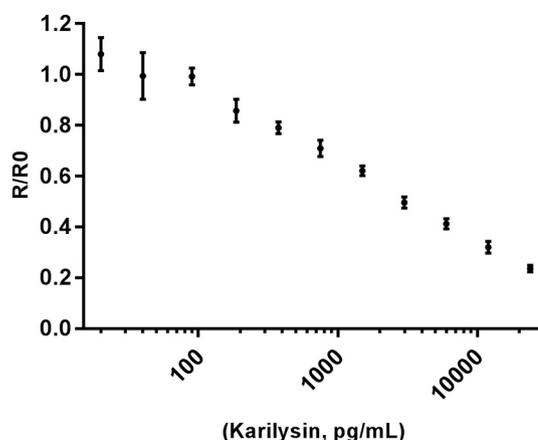


Fig. 4. Calibration curve of the competitive ELISA for quantification of karilysin. Shown are mean and standard deviations from five independent experiments.

Table 2

Performance of the IgY-based karilysin immunoassay. Shown are percentage coefficient of variation (CV %) for intra-day analysis and inter-day analysis.

Concentration (pg/ml)	Intra-assay (CV %) (n = 5)	Inter-day (CV %) (n = 3)
24000	4.7	6.2
12000	6.4	3.8
6000	5.3	5.2
3000	3.8	1.4
1500	3.9	4.9
750	3.8	1.4
375	1.6	1.4
187	3.4	4.6
93	9.0	6.0
46	4.7	1.7
23	6	4.8

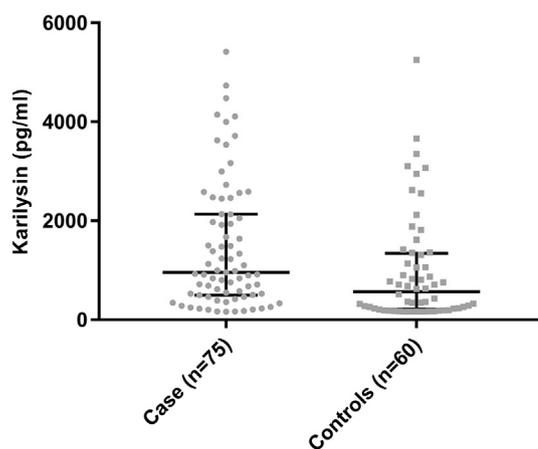


Fig. 5. Karilysin in saliva is detected by the competitive ELISA in periodontitis cases and controls. Shown are concentrations from each subject. The median is shown as well as error bars which represents by the interquartile range.

5. Conclusions

In this study, we used avian IgY technology to create a specific karilysin probe with low nanomolar affinity. This enabled the development a robust IgY-based immunoassay for detection of karilysin in saliva samples from a well-defined study group of adolescents with periodontitis. We found that karilysin is statistically significantly positively associated with signs of periodontitis in this population. These findings can open up a novel avenue of simplified diagnostic research

focused on detection of excreted bacterial biomarkers instead of the bacterial species themselves.

Acknowledgements

PDS, PH and JZK acknowledge financial support from Innovationsfonden grant number 5189-00162B. MK received financial support from Grant “Mobilność Plus” (1306/MOB/IV/2015/O), Polish Ministry of Science and Higher Education. JP was funded by R21DE026280 from NIH/NIDCR and UMO-2016/21/B/NZ1/00292 from National Science Center (Poland).

References

- Bryzek, D., Ksiazek, M., Bielecka, E., Karim, A.Y., Potempa, B., Staniec, D., Koziel, J., Potempa, J., 2014. A pathogenic trace of *Tannerella forsythia* - shedding of soluble fully active tumor necrosis factor alpha from the macrophage surface by karilysin. *Mol Oral Microbiol* 29, 294–306.
- Conroy, P.J., O’Kennedy, R.J., Hearty, S., 2012. Cardiac troponin I: a case study in rational antibody design for human diagnostics. *Protein Eng. Des. Sel.* 25, 295–305.
- Dag, E., Aydin, S., Ozkan, Y., Erman, F., Dagli, A.F., Gurger, M., 2010. Alteration in chromogranin A, obestatin and total ghrelin levels of saliva and serum in epilepsy cases. *Peptides* 31, 932–937.
- Eke, P.I., Dye, B.A., Wei, L., Slade, G.D., Thornton-Evans, G.O., Borgnakke, W.S., Taylor, G.W., Page, R.C., Beck, J.D., Genco, R.J., 2015. Update on prevalence of periodontitis in adults in the United States: NHANES 2009 to 2012. *J. Periodontol.* 86, 611–622.
- Guevara, T., Ksiazek, M., Skottrup, P.D., Cerda-Costa, N., Trillo-Muyo, S., de Diego, I., Riise, E., Potempa, J., Gomis-Ruth, F.X., 2013. Structure of the catalytic domain of the *Tannerella forsythia* matrix metalloproteinase karilysin in complex with a tetrapeptidic inhibitor. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 69, 472–476.
- Imamura, T., 2003. The role of gingipains in the pathogenesis of periodontal disease. *J. Periodontol.* 74, 111–118.
- Jiang, L., He, L., Fountoulakis, M., 2004. Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J. Chromatogr. A* 1023, 317–320.
- Jiao, Y., Hasegawa, M., Inohara, N., 2014. The role of oral pathobionts in dysbiosis during periodontitis development. *J. Dent. Res.* 93, 539–546.
- Jusko, M., Potempa, J., Karim, A.Y., Ksiazek, M., Riesbeck, K., Garred, P., Eick, S., Blom, A.M., 2012. A metalloproteinase karilysin present in the majority of *Tannerella forsythia* isolates inhibits all pathways of the complement system. *J. Immunol.* 188, 2338–2349.
- Karim, A.Y., Kulczycka, M., Kantyka, T., Dubin, G., Jabaiah, A., Daugherty, P.S., Thogersen, I.B., Enghild, J.J., Nguyen, K.A., Potempa, J., 2010. A novel matrix metalloprotease-like enzyme (karilysin) of the periodontal pathogen *Tannerella forsythia* ATCC 43037. *Biol. Chem.* 391, 105–117.
- Koziel, J., Karim, A.Y., Przybyszewska, K., Ksiazek, M., Rapala-Kozik, M., Nguyen, K.A., Potempa, J., 2010. Proteolytic inactivation of LL-37 by karilysin, a novel virulence mechanism of *Tannerella forsythia*. *J. Innate Immun.* 2, 288–293.
- Kyhse-Andersen, J., 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* 10, 203–209.
- Lopez, R., Fernandez, O., Jara, G., Baelum, V., 2001. Epidemiology of clinical attachment loss in adolescents. *J. Periodontol.* 72, 1666–1674.
- Lopez, R., Frydenberg, M., Baelum, V., 2009. Clinical features of early periodontitis. *J. Periodontol.* 80, 749–758.
- Lopez, R., Baelum, V., Hedegaard, C.J., Bendtzen, K., 2011a. Serum levels of C-reactive protein in adolescents with periodontitis. *J. Periodontol.* 82, 543–549.
- Lopez, R., Dahlen, G., Retamales, C., Baelum, V., 2011b. Clustering of subgingival microbial species in adolescents with periodontitis. *Eur. J. Oral Sci.* 119, 141–150.
- Myszka, D.G., 1999. Improving biosensor analysis. *J. Mol. Recognit.* 12, 279–284.
- Schulz, B.L., Cooper-White, J., Punyadeera, C.K., 2013. Saliva proteome research: current status and future outlook. *Crit. Rev. Biotechnol.* 33, 246–259.
- Schwartz, E.B., Granger, D.A., 2004. Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clin. Chem.* 50, 654–656.
- Siddiqi, A., Milne, T., Cullinan, M.P., Seymour, G.J., 2016. Analysis of *P. gingivalis*, *T. forsythia* and *S. aureus* levels in edentulous mouths prior to and 6 months after placement of one-piece zirconia and titanium implants. *Clin. Oral Implants Res.* 27, 288–294.
- Skottrup, P.D., Leonard, P., Kaczmarek, J.Z., Veillard, F., Enghild, J.J., O’Kennedy, R., Sroka, A., Clausen, R.P., Potempa, J., Riise, E., 2011. Diagnostic evaluation of a nanobody with picomolar affinity toward the protease RgpB from *Porphyromonas gingivalis*. *Anal. Biochem.* 415, 158–167.
- Skottrup, P.D., Sorensen, G., Ksiazek, M., Potempa, J., Riise, E., 2012. A phage display selected 7-mer peptide inhibitor of the *Tannerella forsythia* metalloprotease-like enzyme Karilysin can be truncated to Ser-Trp-Phe-Pro. *PLoS One* 7, e48537.
- Skottrup, P.D., Dahlen, G., Baelum, V., Lopez, R., 2018. Soluble urokinase-type plasminogen activator receptor is associated with signs of periodontitis in adolescents. *Eur. J. Oral Sci.* 126 (4), 292–299 (Aug).
- Sochalska, M., Potempa, J., 2017. Manipulation of neutrophils by *Porphyromonas gingivalis* in the development of periodontitis. *Front. Cell. Infect. Microbiol.* 7, 197.
- Socransky, S.S., Haffajee, A.D., Cugini, M.A., Smith, C., Kent, R.L., 1998. Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* 25, 134–144.
- Sorsa, T., Gursoy, U.K., Nwhator, S., Hernandez, M., Tervahartiala, T., Leppilähti, J., Gursoy, M., Kononen, E., Emingil, G., Pussinen, P.J., Mantyla, P., 2016. Analysis of matrix metalloproteinases, especially MMP-8, in gingival crevicular fluid, mouthrinse and saliva for monitoring periodontal diseases. *Periodontol.* 70, 142–163.