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QCM-based rapid detection of PCR amplification products of *Ehrlichia canis*

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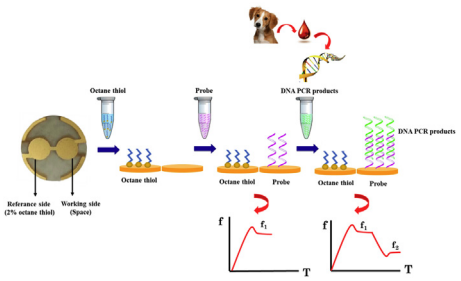
HIGHLIGHTS

- A rapid screening method based on PCR assay combined with quartz crystal microbalance (QCM) for detecting *E. canis*.
- Detection via DNA-DNA hybridization using QCM in situ.
- The technique is simple, does not require complicated equipment and can be reused.
- Immobilizing specific capture probes on the surfaces of QCM is suitable for rapidly detecting DNA of *E. canis*.
- QCM-based DNA sensors turned out sensitive and highly selective against other canine blood parasites or pathogens.

GRAPHICAL ABSTRACT



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ABSTRACT

Ehrlichia canis is an intracellular parasitic bacterium and arthropod-borne pathogen that receives growing attention, because it leads to increasing morbidity and mortality in animals. It does so by causing canine monocytotropic ehrlichiosis (CME). Infected canines may lack obvious clinical signs and stay in chronic stage. Herein we report a rapid screening method based on PCR assay combined with quartz crystal microbalance (QCM) to design a DNA sensor for detecting *E. canis* in early stages of infection. The test relies on DNA amplification of target nucleotide sequences via PCR followed by detecting DNA-DNA hybridization using QCM. The approach did not result in any cross-hybridization toward other blood bacteria or parasites in dogs, such as *Anaplasma platys*, *Babesia canis* and *Trypanosoma* spp, but turned out selective for the target species. The limit of detection of QCM was as low as 4.1×10^9 molecules/μl of 289 bp *E. canis* PCR products corresponding to 22 copy numbers/μl of *E. canis*. Furthermore, the technique is also simple, does not require complicated equipment and can in principle be reused.

1. Introduction

Ehrlichia canis is an obligate intracellular bacterium and causes tick-borne diseases. It has been shown to be transmitted by both nymphal and adult stages of the brown dog tick, *Rhipicephalus*

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sanguineus [1]. *E. canis* spreads worldwide, although its incidence rate within a defined area varies with the presence and density of its tick vectors [2]. *E. canis* infects canines, where it can cause a clinically relevant disease, namely canine monocytotropic ehrlichiosis (CME). CME proves an increasing problem for public health. Multiple outbursts of new tick-transmitted diseases and increased interest in tracing their respective identity has led to better public awareness about such zoonotic infections [3,4]. CME causes death in dogs and other canids, such as wolves or jackals, and deer. Therefore, it is important to rapidly detect it to ensure rapid treatment of infected animals.

The genome of *E. canis* consists of a single circular chromosome containing 1,315,030 nucleotides [5,6]. *E. canis* infects monocytes and macrophages of canines and develops in phagosomal vacuoles in the cytoplasm of cells forming thickly packed colonies termed morulae [7]. Ehrlichiae have developed a mechanism of inhibiting lysosomal fusion in the vacuoles, where they reside. Finally, they are released from infected cells [7]. CME cyclically manifests itself in canines through three stages: The acute stage, 5–20 days after infection, is characterized by fever, depression, anorexia, slight weight loss, dyspnea, thrombocytopenia, anemia, leucopoenia, and hypergammaglobulinemia. It is followed by the subclinical stage showing no clinical signs and only mild thrombocytopenia. Chronic CME is the final stage and manifests itself by hemorrhages, epitasis, edema and symptoms similar to those shown in the acute phase. This phase is often further complicated by superinfections by other microorganisms [8–11]. Overall, this reduces the physical strength of dogs [1,4,6]. While antibiotic treatment of CME with doxycycline is usually effective, it has to start rapidly and accurately before the respective infected canines show onset of the chronic phase, after which the bacterium can no longer be effectively treated.

CME can be diagnosed in a many different ways: The first method is direct visualization of organisms in stained blood smears. However, it is not a routine method, time-consuming and requires a research laboratory [12]. Furthermore, it gives useful results only within 1–2 weeks after infection. Beyond that point it is necessary to apply cell culture. Indirect immunofluorescence assay (IFA) has been widely used for diagnosis. However, IFA may cross-react to different species of *Ehrlichia* bacteria or organisms from other closely related genera [2,13]. PCR can be utilized to detect *E. canis* via amplifying its DNA. This method has been widely used for detection, but requires agarose gel electrophoresis and (toxic) ethidium bromide staining.

Recently, quartz crystal microbalance (QCM) sensors have become an interesting choice for detecting oligonucleotides [14–19] in DNA biosensors. They detect DNA faster and more selectively, than gel electrophoresis followed by staining [20]. Hence QCM-based DNA biosensors are now increasingly developed for diagnostic applications [20]. As a matter of principle, QCM respond to mass change by frequency change due to their piezoelectric properties. Resonant frequencies of usual QCMs are in the MHz range [21]. QCMs have been extensively applied e.g. for elucidating biomolecular mechanisms [20,22–27], in environmental monitoring [21,28], biotechnology [29], food [30], pharmaceutical and industrial sensing [31]. QCM DNA-biosensors rely on immobilization techniques for oligonucleotide probes on the surface of the respective quartz microbalance [20,26]. Hence, this study aims at testing detection of *E. canis* DNA amplified by PCR with Quartz Crystal Microbalance (QCM) biosensor.

2. Experimental

2.1. Chemicals and reagents

All reagents were purchased from Fluka and Merck, respectively. Immobilization buffer (300 mM NaCl, 20 mM Na_2HPO_4, and 0.1 mM EDTA, pH = 7.4) and hybridization buffer (150 mM NaCl, 20 mM Na_2HPO_4, and 0.1 mM EDTA, pH = 7.4) were sterilized by autoclaving at 121° C, 60 Hz for 15 min. Distilled water (dH_2O) was used in all experiments.

2.2. Samples

2.2.1. Genomic DNA extraction

Blood specimens of canines infected with *E. canis* were collected. Genomic DNA was extracted from tissue by using nucleoSpin® blood kit (Macherey-Nagel Inc. Germany) following the protocol provided by the manufacturer. In a nutshell, this comprised of the following steps: pre-lyse sample step by adding Buffer T1 and Proteinase K followed by incubating 1 h at 42° C; lyse sample step by adding Buffer B3 followed by incubating at 56° C for 10 min; binding DNA step by adding 96% ethanol, centrifuging for 1 min at 11,000 × g. After that the sample was washed with Buffer BW and Buffer B5: In the last step, genomic DNA was eluted with distilled water (dH_2O). The genome of *E. canis* 16S rRNA gene DNA was amplified.

2.2.2. PCR product amplification

The forward primer, FEC, and the reverse primer, REC, were designed by using the software package Primer 3 (http://fokker.wi.mit.edu/primer3/input.htm) (Table 1) and synthesized by Sigma-Aldrich (St. Louis, MO, USA). PCR products were amplified from *E. canis* transformation plasmid vector pGEM®-T easy vector. PCR amplification of partial *E. canis* 16S rRNA gene fragment (289 bp) was manipulated in 25 μl containing 22 copy numbers/μl of the plasmid DNA in 1× PCR buffer, 1 μM of each primer, 1.5 mM $MgCl_2$, 2 mM of dNTP, and 1.5U Taq DNA polymerase (Invitrogen®). PCR was performed by using a DNA thermal cycler (MJ Research PTC-200 Peltier thermal cycler). PCR amplification program comprised of pre-denaturation at 95 °C for 5 min, followed by 30 cycles of denaturing for 30 s at 95 °C, annealing 30 s at 52 °C and extension 30 s at 72 °C. Post-extension was achieved by incubating the reaction at 72 °C for 1 min. The amplification product was analyzed by 1.5% agarose gel electrophoresis.

2.3. Oligonucleotide probes

Specific oligonucleotide probes for hybridization with PCR products were designed according to sequences between the FEC and REC regions of the *E. canis* 16s rRNA gene (289 bp) and were labeled with Thiol ($SH\text{-}C_6$) at the 5′ end, which consisted of poly A (Table 1). The Thiol-labeled probes were synthesized by Sigma-Aldrich (St. Louis, MO, USA).

2.4. QCM apparatus and experimental set-up

2.4.1. Screen printing the electrodes

QCMs were produced by screen printing dual electrode structures with brilliant gold paste (Heraeus; 12%) onto 10 MHz AT-cut quartz blanks (diameter 13.8 mm, Great Microtama Industries, Surabaya, Indonesia). Au electrodes exposed to the sample were 5 mm in diameter, those on the opposite face of the quartz 4 mm. After screen printing, electrodes on quartz were burned at 400° C for 4 h to reveal the pure gold surfaces.

2.4.2. QCM apparatus

The QCM DNA sensor systems comprised of inserting the dual gold electrode quartz into a custom-made flow cell made from PDMS, linked with customized oscillator circuit connected to a frequency counter (Agilent 53131A). Only one face of crystal was

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Table 1
Primers/probes used for QCM DNA biosensor.

Primers/probe	Sequences	Numbers of base (bp)
Probe Thiol *E.canis*	5′- Thiol-AAAAA-AAAAA-TGCGCTCGTTGCGGGACTTAA -3′	31
FEC	5′- ACATCATCC CCACCTTCCT -3′	19
REC	5′- ACTCAAAGGAATTGACGGGGAC -3′	22

exposed to sample solutions. The resonance frequency was read out to a computer connected via USB-GPIB interface by a LabView routine [32]. All experiments were performed at room temperature (~25° C). An Agilent 8712ET network analyzer was used to characterize all transducers.

2.5. Immobilization of oligonucleotide probe and QCM assay

Fig. 1 summarizes the steps to produce in-situ QCM-based DNA sensors: First, the gold surface on the reference side of QCM was blocked by drop-coating 5 μl of a solution containing 2% (w/V) octane thiol in heptane onto one of the gold electrodes and incubating for 2 h in a desiccator at room temperature. Then, the QCM was washed with hybridization buffer to remove unbound octane thiol, rinsed with dH_2O, and stored in a desiccator. The frequency change due to immobilization was determined by network analyzer. In a second step, the thiol-modified oligonucleotide probe was dissolved in immobilization buffer (300 mM NaCl, 20 mM Na_2HPO_4, and 0.1 mM EDTA, pH 7.4). Then, 5 μl of that solution was drop-coated onto the working side gold electrode and incubated for 2 h in a desiccator at room temperature. The quartz crystal was washed with hybridization buffer to remove unbound oligonucleotides, rinsed with dH_2O, and stored in a desiccator. The frequency change resulting from immobilization was determined by network analyzer. Furthermore, only QCM showing less than -5dB damping on each electrode were further processed and used for measurements based on experience of the group. QCM containing immobilized oligonucleotide probes were then used for sensor measurements. A typical QCM measurement comprised of injecting around 200μl hybridization buffer into the flow cell and reading out of the oscillator frequencies at the stable base-line signal. Then, solutions containing one part PCR products were mixed with 50 parts hybridization buffer and injected into the measuring cell. After that the solution containing the PCR products was removed, and the cell washed three times with 200 μl of hybridization buffer by flushing roughly through the cell and measured with frequency counter. All steps were monitored via sensor measurements in the oscillator circuit.

The irreversible part of the frequency shift during octane thiol and probe deposition, respectively, corresponds to the amount of immobilized thiol and probe. After this, the irreversible frequency shift during hybridization indicates the amount of PCR products bound to the surface. During washing steps, frequency readout by the software was paused to avoid signal disturbances due to quickly changing flow rate, pressure, or ionic strength.

Sensitivity tests of QCM assay were based on the protocols above using sample solutions containing 22 copy numbers/μl of *E. canis* plasmid template for PCR process. These were serially diluted (1:5 to 1:200) in hybridization buffer, depending on the experiment. Selectivity was assessed by recording the responses towards 30 ng/μl of genomic DNA from *Anaplasma platys*, *Babesia canis*, *Trypanosoma* spp. blood from healthy dogs and negative control.

3. Results

3.1. PCR products

PCR products were synthesized using FEC and REC primers of *E. canis* 16S rRNA gene transformation plasmid as a template. The amplification product was analyzed by 1.5% agarose gel electrophoresis and was 289 bp in size (Fig. 2). This confirms successful amplification.

3.2. Optimization of immobilizing conditions

Fig. 3 summarizes the outcomes of varying two process parameters for immobilizing oligonucleotide probes, namely probe concentration (0.5, 1, 2 and 5 μM), and immobilization time (0.5, 1, 2, 3, 4, and 5 h). Not surprisingly, higher probe concentrations and longer immobilization (up to 2 h) in general lead to higher frequency shifts. Using either 0.5 μM, or 2 μM thiolated probe, respectively, and a reaction time of 2 h in the desiccator turned out optimal.

In a first step it is necessary to clarify if hybridization events can indeed be detected on QCM, i.e. if the devices are sufficiently sensitive. Fig. 4 shows the outcome for this approach: it depicts the frequency responses of a dual-electrode QCM comprising immobilized capture probe for DNA of *E. canis* on one electrode. After exposing this device to a solution containing PCR products of

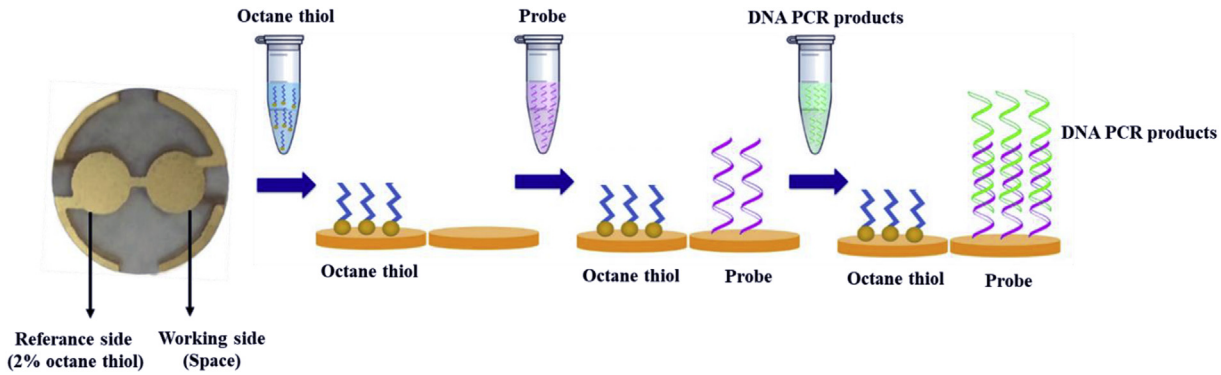

Fig. 1. Scheme of in-situ immobilization of the oligonucleotide probe and QCM assay.

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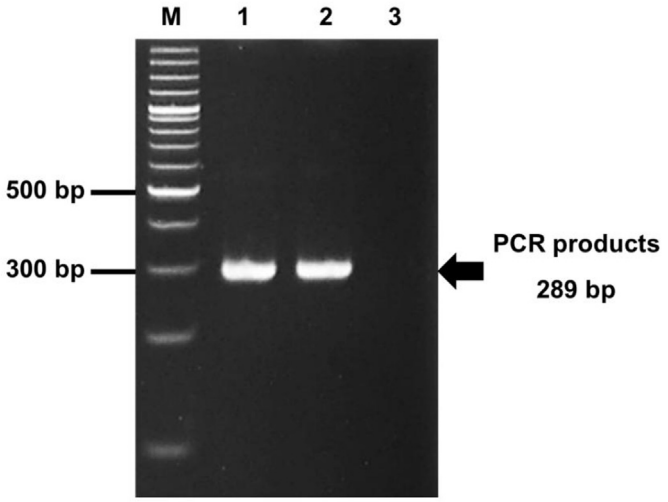

Fig. 2. The 1.5% agarose gel electrophoresis pattern of PCR amplification. Lane M represents 100 bp plus Marker. Lanes 1 and 2 represent PCR products of 289 bp in size. Lane 3 represents negative control.

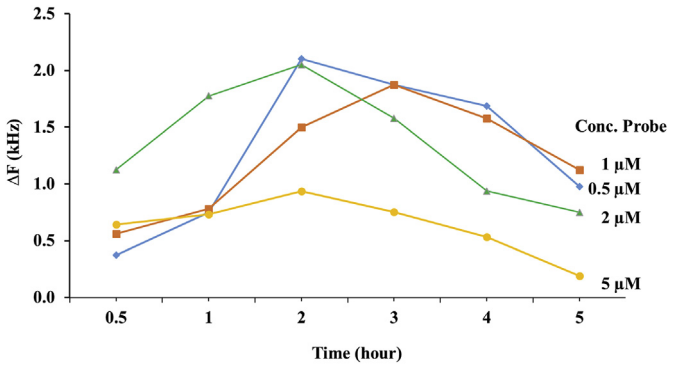

Fig. 3. Optimization of concentrations thiol-modified oligonucleotide probe 0.5, 1, 2 and 5 µM in time of immobilize 0.5, 1, 2, 3, 4 and 5 h. The optimization of thiol-probe was 0.5 µM and 2 µM for 2 h in desiccator.

E. canis genome, both frequencies obviously change: Whereas the frequency on the modified channel shifts by -7.5 kHz, the corresponding effect on the untreated electrode is only around -1 kHz. This strongly indicates that modifying the device surface indeed leads to substantially increased sensitivity. However, two aspects have to be considered in this case: firstly, both sensor signals are obviously reversible, which is not consistent with the nature of DNA double strand binding. Secondly, the corresponding sensor signals are comparably large, namely in the kHz range. The most probable reason for this is that QCM in liquid phase do not yield "pure" mass signals. Their responses also depend on changes in both density/viscosity as modelled by the Kanazawa-Gordon equation, and on changes in surface charge [33]. It is well-known that the DNA backbone is strongly anionic, i.e. contains large number of negatively charged sites. Despite these two shortcomings, the data shown in Fig. 4 clearly demonstrates that immobilizing the capture probe on the QCM electrode increases affinity of the surface toward target DNA. Naturally, this effect requires further in-deep studies to clarify its details.

Thus, the main question remained open, namely if the respective probe indeed had been correctly immobilized. QCM offers the possibility to monitor this process on-line and thus to assess in-situ binding of the probe. Fig. 5 shows the outcome of the approach: the electrode exposed to octane thiol gave rise to a frequency shift of roughly -120 Hz, whereas the probe resulted in -200 Hz. Both effects turned out irreversible and hence clearly demonstrate covalent immobilization of the respective species on the QCM surface. In this setup, octane thiol was used to inactivate the gold surface and to prevent further binding of the probe and ensure proper reference signals.

In the next step, we exposed the sensor to PCR product solution containing 8.2×10^9 molecules/µl *E. canis* DNA. The channel comprising the immobilized probe immediately led to a sensor response of -370 Hz indicating binding of DNA on the device surface. At the same time, no additional response could be observed on the reference side. After reaching equilibrium and flushing with buffer, the frequency on the channel coated with octane thiol remained almost constant. On the probe side the frequency increased by about 72 Hz indicating that roughly only 15% of the initially bound DNA is removed again. Measurements on the reference side had to be stopped somewhat earlier due to electronic problems in the oscillator. However, the signal on the reference side after exposing to target DNA reaches less than 5% of the signal on the probe side and is fully reversible. Hence, Fig. 5 clearly proves feasibility of the approach: around 85% of the *E. canis* PCR products bound to the modified QCM surface irreversibly. This strongly indicates DNA hybridization and double strand formation on the surface. If reversible sensor responses are desired, however, the system has to be washed with dehybridization buffer before reusing it. The other possibility is to use QCM as a proof of concept and to transfer the results to a disposable sensor format. In terms of

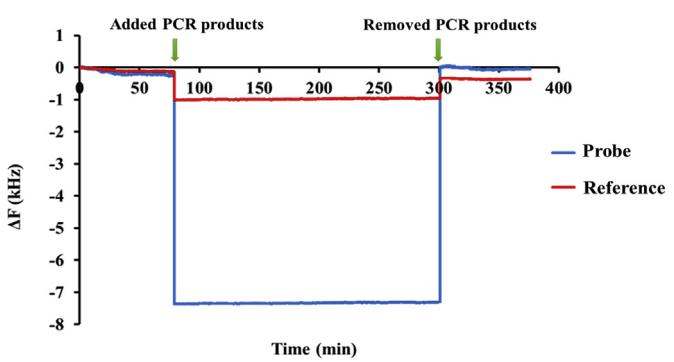

Fig. 4. Frequency change profile of PCR-QCM assay. At minute 79 PCR product sample was added. At minute 301 PCR products sample was removed and the cell rinsed with hybridization buffer.

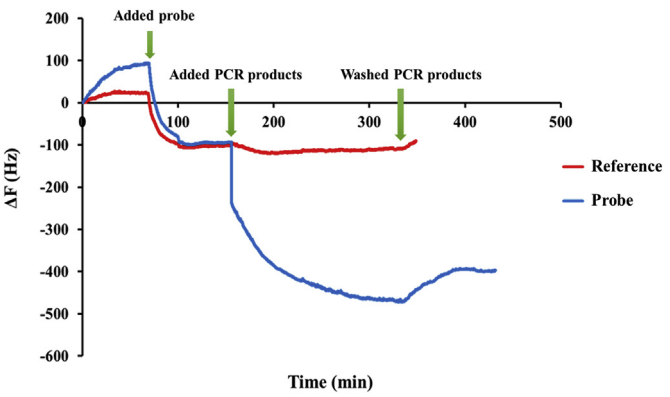

Fig. 5. QCM frequency responses for immobilized probe and thiol-coated reference channel, respectively, after exposure to PCR amplification products. Probe was added in minute 74 followed by PCR product (sample) in minute 155. At minute 338, the quartz was rinsed with hybridization buffer.

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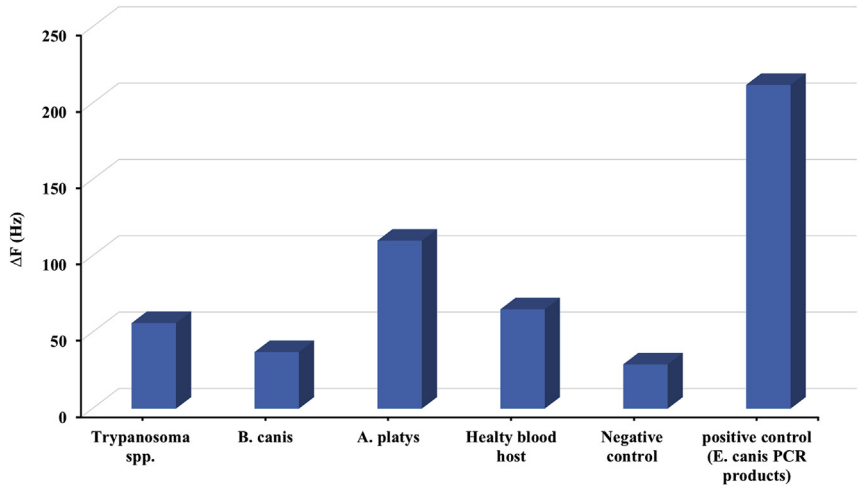

Fig. 6. Selectivity of QCM sensor. No cross-hybridization to host DNA and other blood parasites or bacteria in dog such as *Trypanosoma* spp. *B. canis* and *A. platys*. were observed.

marketability, this seems highly reasonable to avoid cross-contamination between samples. Octane thiol bound to the reference side on the other hand proved unsuitable for binding PCR products, which further underpins its usefulness as a coating for the reference electrode. Overall, Fig. 5 does not only demonstrate that the QCM approach is highly feasible for detecting DNA hybridization, but also its sensitivity: The reason is of course that DNA hybridization is based on hydrogen bonds between the respective nucleobases (2 H-bonds per base pair in A-T, three in C-G). These can reach and exceed the binding energies of covalent bonds, such as the one between the gold electrode and the Sulphur atom of the probe and octane thiol, respectively. For practical purposes, this is of no importance, because the two bonds are cleaved by substantially different strategies making it possible to regenerate the sensor surfaces. Nonetheless, sensitivity alone is of course not enough: for the actual use as a sensor, the system also has to be sufficiently selective.

3.3. Selectivity of QCM biosensor

Fig. 6 summarizes selectivity behavior of the QCM-based DNA-sensor by plotting the difference in irreversible frequency shifts of the QCM against the respective species. Obviously, the positive control leads to large signal, namely around -212 Hz. Surprisingly, negative control reached about 15% of that signal indicating substantial nonspecific effects in the system. All samples containing DNA of other blood parasites or bacteria, namely *Trypanosoma* spp. *B. canis* and *A. platys*. lead to signals that are in the range of negative control, or slightly above it. This clearly demonstrates that actual selective signal is generated by the respective probe DNA.

3.4. Sensitivity of QCM biosensor

Table 2 summarizes the reversible frequency shifts obtained for two different concentrations of *E. canis* DNA, namely 8.2×10^{10} and 4.1×10^{9} molecules/μl. Obviously, the sensor signal obtained for the larger concentration is only about 30% higher, than the one for the lower. This on the one hand shows that these concentrations already lead to signal saturation. In terms of application, however, this is no problem: It is well-known that PCR is not useful for strict quantification, because one cannot know the efficiency of individual amplification steps. The lower concentration, however, is in line with the clinical limit of detection, which is 4.1×10^{9} molecules/μl of 289 bp *E. canis* PCR products corresponding to 22 copy numbers/μl of *E. canis*.

Not being able to fully quantify *E. canis* with this method does not limit its usefulness: such screening tests aim at giving information on the absence or presence of the respective pathogen to allow for corresponding therapeutic measures. However, they usually do not require to exactly quantify pathogen concentrations in blood.

Table 2
Sensitivity test of QCM assay for of *E. canis* PCR products.

Concentration of *E. canis* PCR products (molecules/μl)	Δf (Hz)
8.2×10^{10}	−204
4.1×10^{9}	−269

4. Conclusion

We successfully demonstrated that immobilizing specific capture probes on the surfaces of QCM is suitable for rapidly detecting DNA of *E. canis*. Detection is based on specific hybridization of thiol-modified oligonucleotide probe comprising specific sequence of *E. canis* 16s rRNA gene. The respective sensor turned out highly selective against other canine blood parasites or pathogens, such as *A. platys*, *B. canis* and *Trypanosoma* spp. Overall, the setup hence shows substantial potential for such DNA-based pathogen screening tests.

Acknowledgements

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