Detection and quantification of *Erysipelothrix rhusiopathiae* in
blood from infected chickens – addressing challenges with
detection of DNA from infectious agents in host species with
nucleated red blood cells

Eva Wattrang1,*, Victoria Jäderblom1, Tomas Jinnerot1, Helena Eriksson2, Elisabeth Bagge1,2, Maria Persson2, Tina
Sørensen Dalgaard3 and Robert Söderlund1,*

**Abstract**

**Purpose.** The present study aimed to establish pretreatment protocols as well as real-time and droplet digital polymerase
chain reaction (PCR) methodologies to detect and quantify *Erysipelothrix rhusiopathiae* (ER) DNA in blood samples from infected
chickens, as tools for routine diagnostics and monitoring of experimental infections. Chicken blood is a problematic matrix for
PCR analysis because nucleated erythrocytes contribute large amounts of host DNA that inhibit amplification.

**Methodology.** Using artificially spiked samples of fresh chicken blood, as well as blood samples from three experimental infec-
tion studies, the performance of pretreatment protocols, including choice of blood stabilization agent, centrifugation speeds
and Ficoll gradient separation, was evaluated. The results were compared with those from traditional culture-based protocols
combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

**Results/Key findings.** Simple preparations producing cell-free samples performed well on artificial spike-in samples, provid-
ing high sensitivity. However, performance was poor in clinical samples or artificial samples where the bacteria were incubated
for 4 h or more in fresh blood prior to DNA extraction. In these samples, a Ficoll separation protocol that creates samples rich
in lymphocytes, monocytes and thrombocytes prior to DNA extraction was far more effective.

**Conclusions.** Our results indicate that ER bacteria undergo rapid phagocytosis in chicken blood and that analysis of a blood
fraction enriched for phagocytic cells is necessary for reliable detection and quantification. The presented results explain the
poor performance of PCR detection reported in previously published experimental ER infection studies, and the proposed solu-
tions are likely to have broader implications for PCR-based veterinary diagnostics in non-mammalian host species such as
poultry and fish.

**INTRODUCTION**

Blood samples are routinely used for a wide range of tests in
both human and veterinary infectious disease diagnostics. While culture-dependent tests are still commonly used in
bacteriology, techniques based on polymerase chain reac-
tion (PCR) detection of pathogen DNA have been widely
adopted for blood samples, as they are fast and cost-effective.
Several components in blood, including haemoglobin, can
inhibit PCR reactions [1], although in general this can easily
be overcome with standard DNA extraction protocols. It
has also been repeatedly observed that the presence of large
quantities of host DNA or other irrelevant DNA in a sample
can inhibit PCR [2, 3], as well as the related technique of
droplet digital PCR (ddPCR) [4, 5]. The presence of host DNA
is usually not an issue in the analysis of mammalian blood,
since erythrocytes are by far the most common host cell type
in these samples, and mammalian erythrocytes generally
lack a nucleus and contain little or no DNA. Leukocytes are nucleated and also present in blood, but are far less numerous. In contrast, the erythrocytes of most non-mammalian vertebrates do contain a nucleus, and blood samples will thus contain very large quantities of host DNA. For example, the blood of both humans and chickens contains in the order of $10^{12}$ erythrocytes and $10^9$ leukocytes $l^{-1}$ [6, 7], and the amount of host DNA is therefore in the order of a 1000-fold higher per volume unit in chicken blood compared to human blood. This poses a diagnostic challenge when analysing blood samples from chickens, as well as fish [8] and other non-mammalian vertebrates, with PCR. Unless host and pathogen cells can be separated in a preliminary stage, the inhibition can only be resolved by diluting the sample, resulting in an obvious loss of sensitivity. Metagenomic sequencing approaches to diagnostics hold great promise for the future beyond PCR [9], but they are even more sensitive to the presence of excessive host DNA.

In modern egg production, the disease erysipelas, caused by the Gram-positive bacterium *Erysipelothrix rhusiopathiae* (ER), is an increasing problem, especially in cage-free housing systems, including systems where hens have access to the outdoor environment, e.g. free range and organic production [10–16]. The disease manifests as outbreaks with high mortality (up to 60%) and egg production losses. The affected chickens display acute septicaemia and diagnosis is made through pathological findings in combination with isolation of ER from liver or spleen [15, 16]. Diagnostic culture of ER from necropsy samples or non-aseptically collected blood samples involves culture in selective media and takes 2–4 days, depending on the contamination of samples [16]; aseptically collected blood samples may shorten it to 2 days, but can be difficult to achieve in the field. Hence, it would be of great value if this process could be shortened by applying PCR methodology to detect ER DNA in chicken specimens. If such a protocol were applied directly to blood samples from affected flocks, a diagnosis could be made within 24 h. Therefore, the aim of the present study was to establish sensitive real-time PCR and ddPCR assays for the detection of ER DNA in chicken blood samples.

**METHODS**

**Bacterial strains**

The ER strains 16-BKT031015 and 15-ALD003475, both derived from laying hens with clinical disease and *Erysipelothrix tonsillarum* strain CCUG31352, were stored at $-70^\circ$C and if not otherwise stated, bacteria were cultured on horse blood agar (National Veterinary Institute, Uppsala, Sweden) for 48 h at $37^\circ$C before analysis.

**DNA extraction**

DNA was extracted from bacterial preparations or different blood sample preparations (see below) by suspension in 100 $\mu l$ water (Sigma-Aldrich), heating to 100 $^\circ$C for 15 min and cooling at $-20^\circ$C for 10 min. Thereafter debris was removed by centrifugation at 10 000 $g$ for 10 min and the supernatant as collected and stored at $-70^\circ$C until analysis.

**Quantitative real-time PCR assay**

The primers and the ER-specific probe detecting the noncoding region downstream of the 5S rRNA coding region described earlier [17] were synthesized by Eurofins Genomics and used throughout this study. Real-time PCR reactions had a total volume of 15 $\mu l$ containing 7.5 $\mu l$ PerfeCTa qPCR ToughMix (QuantaBio), 0.4 $\mu M$ of each primer, 0.13 $\mu M$ of probe and 2 $\mu l$ of DNA samples.

Reactions were carried out in an ABI 7500 Fast Real-Time PCR system (Thermo Fisher Scientific) using MicroAmp Fast Optical 96-Well Reaction Plates (Thermo Fisher Scientific). The PCR cycling parameters were 3 min at 95 $^\circ$C, followed by 45 cycles of 3 s at 95 $^\circ$C and 30 s at 60 $^\circ$C. The results were analysed with ABI 7500 Fast system software v 2.3 (Thermo Fisher Scientific). The threshold for each run was set at 0.2 to eliminate background noise before calculation of the cycle when the fluorescence threshold was reached ($C_T$ value) for each positive sample. DNA from *E. tonsillarum* was used as negative DNA control. The bacterial DNA concentrations from each bacterial species, measured with the Qubit QuantIT HS assay, together with the predicted genome size for each species, were used to calculate the genome copy number $\mu l^{-1}$. The ER DNA was diluted 1 : 10 in seven steps, from $2 \times 10^9$ to 2 copies per reaction. Each dilution was analysed in triplicate to create a standard curve and confirm the expected sensitivity of the assay. Each sample from spike-in and experimental infection experiments was tested in duplicate and all DNA samples were analysed undiluted. In addition, samples from infected chickens in infection trial 3 were also analysed diluted 1 : 10 in water prior to addition to the PCR reaction. Each assay included a positive DNA control consisting of $2 \times 10^9$ genome copies of the ER strain 16-BKT031015 and a negative no-template control (NTC) with the sample volume replaced by water.

**Droplet digital PCR assay**

The same primers and probe as in the real-time PCR were used in the ddPCR. This assay was set up according to the Droplet Digital PCR Applications Guide (Bio-Rad; http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf). In brief, the ddPCR reactions had a total volume of 20 $\mu l$ containing 10 $\mu l$ ddPCR Supermix for Probes (Bio-Rad) 0.53 $\mu M$ of each primer, 0.17 $\mu M$ of probe and 2 $\mu l$ of DNA samples. Droplets were generated in a QX100 Droplet Generator (Bio-Rad) and PCR reactions were carried out in a thermal cycler with PCR cycling parameters of 10 min at 95 $^\circ$C followed by 40 cycles of 30 s at 94 $^\circ$C and 60 s at 57.8 $^\circ$C, followed by 10 min at 98 $^\circ$C. After the amplification step, the droplets were analysed in a QX100 Droplet Reader (Bio-Rad) and the data were analysed using QuantaSoft software (v 1.5.38.1118). FAM fluorescent droplets were analysed in channel 1 and fluorescent and non-fluorescent droplets were separated with the threshold set at 4000 AU (Fig. 4). Samples with less than three positive droplets were considered
to be negative. With this criterion and the dilution factors for analysing blood samples, the detection limit of the assay was approximately $1 \times 10^4$ copies ml$^{-1}$ blood.

**Preparation of ER-spiked chicken blood samples**
The ER strain 15-ALD003475 was cultured for 48 h at 37 °C in meat broth (National Veterinary Institute). Subsequently, a 10-fold serial dilution of the bacterial suspension was performed and 100 μl volumes of each dilution were spread on agar plates and cultured for 48 h at 37 °C, and ER colonies were counted and colony-forming units (c.f.u.) ml$^{-1}$ suspension were calculated. The bacterial suspension was mixed with EDTA-stabilized chicken blood from a good health blood donor layer flock (Håtunabalb) to achieve concentrations of 10000, 1000 and 100 c.f.u. ml$^{-1}$ blood, respectively, and blood samples were immediately prepared for DNA extraction according to protocol A (see below).

**Incubation of ER in chicken blood**
A suspension of ER strain 15-ALD003475 cultured in meat broth was centrifuged at 10 000 g for 10 min and the pellet was resuspended in phosphate-buffered saline (PBS; pH 7.0, without Ca$^{2+}$ and Mg$^{2+}$). This bacterial suspension was added to either EDTA or heparin-stabilized chicken blood (Håtunabalb) at ratios of 1:6 and 1:4, respectively. Each blood preparation was aliquoted into two samples that were incubated at 40 °C for either 4 or 16 h. After incubation, each sample was divided into three and prepared for DNA extraction according to protocols A, B or C, respectively (see below).

**Preparation of chicken blood samples for DNA extraction**
**Protocol A – ‘cell-free fraction’ (CFF)**
Chicken blood samples were processed as previously described [18]. Cells were sedimented by centrifugation at 1700 g for 1 min and the supernatant was collected and centrifuged at 10 000 g for 10 min, after which it was discarded and the pellet was then stored at −80 °C until DNA extraction. In the case of the experimentally infected chickens in infection trials 2 and 3, 220 μl EDTA-stabilized blood was processed from each sample.

**Protocol B – ‘slow-speed centrifugation’ (SSC)**
Blood samples were diluted with an equal volume of PBS and centrifuged at 60 g for 15 min without brake in a swing-out rotor, and the plasma phase and the leukocyte layer above the red blood cells were collected and centrifuged at 10 000 g for 10 min, after which the supernatant was discarded and the pellet was then stored at −80 °C until DNA extraction. In the case of the experimentally infected chickens in infection trials 2 and 3, 220 μl EDTA-stabilized blood was processed from each sample.

**Protocol C – ‘Ficoll separation’ (FS)**
Blood samples were diluted with an equal volume of PBS and layered onto 1 ml Ficoll-Paque PLUS (GE Healthcare Life Sciences) and centrifuged at 400 g for 25 min without brake in a swing-out rotor. The interphase cells and all plasma above were collected and centrifuged at 10 000 g for 10 min, after which the supernatant was discarded and the pellet was then stored at −80 °C until DNA extraction. In the case of the experimentally infected chickens in infection trials 2 and 3, 220 μl EDTA-stabilized blood was processed from each sample.

**Experimental ER infection of chickens**
The study comprised three separate infection trials with a total of seven groups of ER-infected SPF chickens ($n=19$–$13$ chickens/group) and one group ($n=13$) of uninfected chickens. The chickens, housing, trial designs and experimental procedures are described in detail in the Supplementary Material. In brief, the chickens were infected by intra-muscular injection of ER of the 15-ALD003475 strain on experimental day 0 and blood samples for serum were collected by jugular venipuncture from chickens on the indicated experimental days.

**Culture of ER in blood samples from infected chickens**

**Selective sodium azide/crystal violet broth**
Immediately after blood collection, 10 μl unstabilized blood was transferred with a disposable cultivation loop to 5 ml selective sodium azide/crystal violet broth (National Veterinary Institute; containing 5 μg ml$^{-1}$ crystal violet and 0.2 mg ml$^{-1}$ sodium azide) and incubated for ER enrichment for 48 h at 37 °C. Then 10 μl of broth was spread on horse blood agar plates and incubated for 48 h at 37 °C. The identity of suspected ER colonies was subsequently verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Biotyper instrument (Bruker). This method was used on blood samples collected in infection trials 1 and 2.

**Direct culture**
EDTA-stabilized blood samples were 10-fold diluted with PBS in three steps and 100 μl of undiluted blood and 1:100 and 1:1000 dilutions, respectively, were added to horse blood agar plates and distributed evenly with plastic spreaders and incubated at 37 °C for 48 h before the counting of suspected ER colonies, based on morphology, and the identity of these colonies was subsequently verified by MALDI-TOF MS. This method was used for blood samples collected in infection trials 2 and 3.

**RESULTS**

**Evaluation of the ER real-time PCR assay**
DNA from ER strain 16-BKT0310115 derived from a laying hen with clinical disease was used to determine the sensitivity of a previously published real-time PCR assay used for the detection of ER in tissue samples and oral swabs from pigs [17] and to calculate the amplification efficiency. The
presence of ER was reliably detected at the level of 20 copies/reaction, with all replicates being positive, but it could not be reliably detected at the level of 2 copies/reaction, indicating that the detection limit is close to optimal in DNA samples from cultured bacteria. The amplification efficiency, $E$, was calculated from the slope of the standard curve as $E=10^{-1/\text{slope}}$, to 98.5%. The generated standard curve was further used to approximate the number of genome copies in clinical samples.

To evaluate the assay for inclusivity in terms of detecting genetically diverse types of ER, as well as exclusivity in terms of ability to distinguish ER from closely related bacteria, 10 diverse ER isolates representing the known clades of ER [19] and 3 *E. tonsillarum* isolates were selected. All ER isolates were positive, while all three *E. tonsillarum* isolates were negative.

Analysis of chicken blood samples ‘spiked’ with ER with blood samples prepared using protocol A, CFF, showed that the assay readily detected DNA from 10 000 to 100 c.f.u. ml$^{-1}$ blood.

**Experimental ER infection of chickens – infection trial 1**

In this trial, three groups of chickens, A, B and C, were infected with $0.5 \times 10^5$, $0.5 \times 10^6$ or $0.5 \times 10^7$ c.f.u. ER/chicken, respectively (Table S1, available in the online version of this article). One chicken in group B showed mild signs of depression on day 4 after infection. No clinical signs of disease were observed among the other chickens during the experiment. Through culture of blood in sodium azide/crystal violet broth, growth of ER was detected in a total of 10 samples collected between days 1 and 5 after infection (Fig. 1a, Table S1). However, when using blood preparation protocol A, CFF, before DNA extraction, only one blood sample was positive for ER DNA (Fig. 1a, Table S1); $2.1 \times 10^4$ genome copies ml$^{-1}$ blood by real-time PCR and $6.8 \times 10^3$ genome copies ml$^{-1}$ blood by ddPCR, respectively. This sample was indeed also positive for ER growth.

Thus, it seemed that the real-time PCR was less sensitive in detecting ER DNA in samples from infected chickens than when using artificially spiked samples.

**Evaluation of different methods for blood preparation before DNA extraction**

To test whether blood preparation methods that include leukocytes in the DNA extraction could enhance the yield of ER DNA from blood samples, live bacteria were incubated at 40 °C with freshly collected chicken blood to allow bacterial entry in to cells and/or bacterial uptake by leukocytes. After incubation, blood samples were prepared for DNA extraction in parallel using three different protocols: A – CFF; B – SSC; C – FS. Protocols B and C both included leukocytes, but protocol C resulted in the inclusion of a larger fraction of thrombocytes.

After 4 h of incubation, more than 30 times more ER DNA was detected in blood samples prepared using FS compared to CFF or SSC (Fig. 2). After 16 h of incubation, no or very small amounts of ER DNA were detected in blood samples
prepared using CFF or SSC, while samples prepared with FS were strongly positive. There were some differences between the amounts of DNA detected in heparin- compared to EDTA-stabilized blood, but no clear influence of stabilizer was observed within this limited dataset.

Thus, it seems that ER may quickly enter/become phagocytosed in blood cells during in vitro mimicking of physiological conditions and that blood preparation by FS that includes leukocytes with a large proportion of thrombocytes increased the yield of ER DNA detected by PCR.

**Experimental ER infection of chickens – infection trial 2**

In this trial, two groups of chickens, D and E, were infected with 1.6×10⁸ or 1.6×10⁶ c.f.u. ER/chicken, respectively (Table S2). None of the chickens showed any clinical signs of disease during the experiment. Growth of ER was detected in a total of six samples collected on days 3 or 5 after infection by direct culture of blood (Fig. 1b, Tables S2 and S3). Growth of ER was only detected in four of these samples by culture of blood in sodium azide/crystal violet broth (Tables S2 and S3). Aliquots of all blood samples were also prepared according to protocol C, FS, prior to DNA extraction and analysed for ER DNA using real-time PCR. This analysis showed bacterial DNA in four of the six culture-positive samples on days 3 and 5 and in two samples that were negative for bacterial culture on day 10 (Fig. 1b, Tables S2 and S3). Samples that were positive for ER by either culture or real-time PCR were also analysed for ER DNA by ddPCR (Fig. 3, Table S3). In this infection trial, two of the samples that were positive by either culture or real-time PCR were also positive in the ddPCR. These two samples showed a good correlation between the amount of DNA detected by the two methods (real-time PCR vs ddPCR; 4.1 vs 2.1 and 2.6 vs 1.3, respectively, 10⁴ copies ml⁻¹ blood). The approximate amounts of DNA detected in the samples that were only positive for ER DNA by real-time PCR were below the expected detection limit of the ddPCR.

Hence, the results indicated that the direct blood culture method was more sensitive than culture in selective medium and that the blood preparation protocol using FS increased the detection of ER DNA in culture-positive samples from
infected chickens compared with the CFF protocol used in infection 1.

**Experimental ER infection of chickens – infection trial 3**

This trial comprised three groups of chickens: group F (uninfected), group G (naïve infected) and group H (vaccinated and infected) (Table S4), and the chickens in groups G and H were infected with $0.5 \times 10^{10}$ c.f.u. ER/chicken. One chicken in group G showed moderate signs of depression on days 2 to 4 after infection and did not gain weight during these days. This chicken also had the highest quantity of ER in blood, $10^6$ c.f.u. ml$^{-1}$, when sampled on day 3 (Table S5). No clinical signs of disease were observed for the other chickens during the experiment. Growth of ER was detected in a total of eight signs of disease were observed for the other chickens during infection trials 2 and 3. Only samples that were deemed positive by both methods are shown and on those occasions where DNA samples were positive when both undiluted and in dilution, 1 : 10 results from the 1 : 10 dilution are shown. For comprehensive results, see Tables S3 and S5.

DISCUSSION

The study objective to set up a methodology to detect and quantify ER DNA in blood samples from infected chickens was met with some challenges. In our first experimental ER infection of chickens only 1 of the 10 culture-positive blood samples was positive for ER DNA, even though validation of the real-time PCR showed that it detected DNA from low quantities of ER when the bacteria were mixed with chicken blood and the DNA was isolated immediately. Similar issues were evident in the study by Harada *et al.* [18], with a clear discrepancy between the high sensitivity of PCR when detecting DNA in spiked blood samples and the 100-fold lower detection of DNA in blood samples from infected chickens. The blood preparation protocol used in both studies eliminates the whole host cellular fraction before DNA extraction [18] and hence avoids the problem with excessive amounts of chicken DNA from the red blood cells, which should work well provided that all bacteria are extracellular. However, ER may survive and even proliferate in murine and porcine phagocytic cells (reviewed in [20]). Thus, we hypothesize that ER may persist in chicken phagocytic leukocytes. Some of the bacteria detected by culture may have been intracellular or adherent to cells and therefore lost during blood preparation for PCR, which would explain the discrepancy between culture and PCR results.

To test this hypothesis and evaluate if the blood sample preparation method prior to DNA extraction could be improved by the inclusion of leukocytes, we performed an *in vitro* pilot experiment. By culturing live ER in chicken whole blood, bacterial entry into cells and/or phagocytosis of bacteria by leukocytes could take place *in vitro* before two blood preparation protocols maintaining leukocytes, i.e. SSC and FS, were applied in addition to the CFF method. Our results showed that after 4 h of incubation higher amounts of

![Fig. 3. Correlation between c.f.u. of ER detected by culture and genome copies of ER DNA detected by real-time PCR (red filled circles) or by ddPCR (crosses) in blood from experimentally ER infected chickens in infection trials 2 and 3. Only samples that were deemed positive by both methods are shown and on those occasions where DNA samples were positive when both undiluted and in dilution, 1 : 10 results from the 1 : 10 dilution are shown. For comprehensive results, see Tables S3 and S5.](image-url)
ER DNA was detected in samples prepared by FS compared to SSC and CFF, respectively, and after 16 h of incubation, the samples prepared by FS remained clearly positive, while no or very small amounts of ER DNA were detected in the other samples. Ficoll gradient separation results in the enrichment of lymphocytes, monocytes and thrombocytes from chicken blood, while SSC gives a relatively pure lymphocyte population with only small amounts of thrombocytes [21]. In addition, the proportion of monocytes has been reported to be lower after SSC compared to FS [22]. Chicken blood contains three main populations of phagocytic leukocytes, namely heterophils, monocytes and thrombocytes [23–25]. Thus, it seems that the protocol that includes the most phagocytic cells also resulted in the highest amounts of ER DNA. Hence, these results indicate that ER may indeed be taken up by chicken phagocytic leukocytes in this pilot experiment we used both EDTA- and heparin-stabilized blood. EDTA was suggested as a preferred anticoagulant, since heparin may inhibit PCR analysis [26], although EDTA has also been found to inhibit PCR reactions and it has also been suggested that the choice of anticoagulant may be less critical [27]. Moreover, the chelating properties of EDTA are considered to inhibit phagocytosis, e.g. by inhibiting calcium signalling [28]. However, it has also been show that in vitro phagocytosis can be observed in human whole blood cultures using EDTA-stabilized blood [29]. In the current limited dataset, both PCR reactions and phagocytosis seemed to work equally well in either anticoagulant, although the lower level of ER DNA recovered after FS of samples incubated for 16 h in EDTA- compared to heparin-stabilized blood may have been due to lower long-term viability of leukocytes in the former anticoagulant.

Ficoll separation was therefore applied to blood samples from ER-infected chickens in trials 2 and 3. In infection trial 2, a low proportion of infected chickens showed bacteria in the blood by culture, but with the exception of one sample with only 20 c.f.u. ml−1 blood, ER DNA was detected in all culture-positive samples. Thus, the real-time PCR showed the expected sensitivity when FS was performed prior to DNA extraction from blood samples. These results also indicated that ER may be intracellular in clinical samples. However, in

![Graph showing ddPCR results](image-url)
Infection trial 3, some of the culture-positive samples from day 3 post-infection were negative in the PCR when the undiluted DNA template was used. When the DNA template was diluted 1:10, some of these samples were found to be positive for ER DNA. Hence, it seemed that these DNA samples contained PCR-inhibiting substances, and the effect of these was overcome by dilution of the samples, although this regrettably reduced the sensitivity of the method. During this phase of the infection, the blood leukocyte counts in the infected chickens increased by approximately sixfold (Wattrang et al., in preparation), which consequently would increase the amount of chicken DNA in the samples, and it seems likely that this DNA caused the observed inhibition.

Thus, running diluted samples in parallel can clearly improve the overall detection rates. Because several blood parameters were analysed in parallel, we were restricted in the amount of blood (220 μl) that was available for DNA extraction in the present experiment, which in turn limited the sensitivity of the PCR assays. However, on a single occasion one may easily collect 2 ml blood from a mature chicken, and if this was solely used for DNA extraction, the sensitivity of the PCR analysis could potentially be further improved.

Digital PCR, which is frequently implemented as ddPCR, as in the present study, is more laborious than real-time PCR and is therefore a less attractive option for diagnostics. However, ddPCR is generally considered to be a superior method for quantification and as a result of this provides valuable information, e.g. for following the progression of an infection. However, as previously observed by the authors and others, ddPCR can produce a small number of false-positive observations in any sample, which limits its ability to reliably detect and quantify very low target counts. When ddPCR was applied to samples from the infected chickens, ER DNA was readily detected in samples with large enough amounts of bacterial DNA for this assay and produced similar values for bacterial genome counts compared to real-time PCR, and also comparable c.f.u. values to those from traditional culture, offering a method for good quantification of DNA. Moreover, ddPCR also detected DNA in a few samples containing inhibitory substances that had not been detected in the real-time PCR without dilution, indicating that ddPCR had a slightly higher tolerance of inhibition.

In the present study, sensitive real-time PCR also detected ER DNA in some culture-negative samples. All of these samples were collected during the later stages of infection (day 10 in infection trial 2 and day 5 in infection trial 3), and most were from chickens that had tested positive for ER by culture at earlier sampling occasions. Thus, it seems likely that this ER DNA represents relic DNA [30] remaining in the host system after the bacteria have been killed, e.g. in the bloodstream or inside phagocytic cells.

In conclusion, in the present study we show that the combination of nucleated erythrocytes and rapid host phagocytosis of ER bacteria creates a need for appropriate pretreatment of chicken blood samples for the reliable detection and quantification of the pathogen. Combined culture and PCR-based methods provide optimal sensitivity, with culture being more likely to detect early infection, while samples are more likely to be PCR-positive in the later stages of ER infections. Some PCR inhibition by chicken DNA may occur during the acute phase of the infection, but at flock level, by including blood samples from a number of individuals, it should be possible to use this PCR methodology to rapidly diagnose the disease during an outbreak. We propose that the observed problems and suggested solutions have broader implications for the PCR detection of blood-borne intracellular pathogens – including, for example, viruses and protozoa, in addition to bacteria – in host species with nucleated erythrocytes.

Funding information
The study was supported by the EryPoP project financed by Animal Health and Welfare ERA-Net (ANIHWA) under the European Union Seventh Framework Network (ID number 119, in Sweden grant number 221-2015-1895, in Denmark 5192-00004B) and the Swedish Research Council Formas (grant number 942-2015-766). The funding agencies had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Acknowledgements
The authors wish to thank the staff at the animal facilities at the National Veterinary Institute for excellent animal care, and Osama Ibrahim, Malin Boyner and Anna Lundén for expert technical assistance.

Conflicts of interests
The authors declare that there are no conflicts of interest.

Ethical statement
Animal experiments were approved by the Uppsala Regional Ethical Committee for Animal Experiments, permit no. C46/16.

References
1. Sidsdeth M, Hedman J, Romsons EL, Waitara L, Wadsö L et al. Inhibition mechanisms of hemoglobin, immunoglobulin G, and whole blood in digital and real-time PCR. Anal Bioanal Chem 2018;40:2159–2153.
2. Morata P, Queipo-Ortuño MI, de Dios Colmenero J. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. J Clin Microbiol 1998;36:2443–2446.
3. Cogswell FB, Bantar CE, Hughes TG, Gu Y, Philipp MT. Host DNA can interfere with detection of Borrelia burgdorferi in skin biopsy specimens by PCR. J Clin Microbiol 1996;34:980–982.
4. Strain MC, Lada SM, Luong T, Rought SE, Gianella S et al. Highly precise measurement of HIV DNA by droplet digital PCR. PLoS One 2013;8:e55943.
5. Doescher A, Loges U, Petershofen EK, Müller TH. Evaluation of droplet digital PCR for quantification of residual leucocytes in red blood cell concentrates. Vox Sang 2017;112:744–750.
6. Samour J. Diagnostic value of hematology. In: Harrison G, Light-foot T (editors). Clinical Avian Medicine. Brenthwood: Avian Medicine online; 2018: pp. 587–610.
7. Hoffman R, Silberstein LE, Weitz JI, Salama ME, Benz EJ et al. Hematology. Amsterdam: Elsevier; 2018.
8. Pascho RJ, Elliott DG, Chase DM. Comparison of traditional and molecular methods for detection of Renibacterium salmoninarum. In: Cunningham CD (editor). Molecular diagnosis of salmonid diseases. Dordrecht: Kluwer Academic Publishers; 2002. pp. 157–209.
9. Dekker JP. Metagenomics for clinical infectious disease diagnostics steps closer to reality. J Clin Microbiol 2018;56:e00850–00818.
10. Mazaheri A, Lierz M, Hafez HM. Investigations on the pathogenicity of Erysipelothrix rhusiopathiae in laying hens. Avian Dis 2005;49:574–576.
11. Kaufmann-Bart M, Hoop RK. Diseases in chicks and laying hens during the first 12 years after battery cages were banned in Switzerland. Vet Rec 2009;164:203–207.
12. Fossum O, Jansson DS, Etterlin PE, Vågsholm I. Causes of mortality in laying hens in different housing systems in 2001 to 2004. Acta Vet Scand 2009;51:1–9.
13. Stockholm NM, Permin A, Bogsaa M, Christensen JP. Causes of mortality in commercial organic layers in Denmark. Avian Dis 2010;54:1241–1250.
14. Eriksson H, Brännström S, Skarin H, Chirico J. Characterization of Erysipelothrix rhusiopathiae isolates from laying hens and poultry red mites (Dermanyssus gallinae) from an outbreak of erysipelas. Avian Pathol 2010;39:505–509.
15. Eriksson H, Nyman AK, Fellström C, Wallgren P. Erysipelis in laying hens is associated with housing system. Vet Rec 2013;173:18.
16. Eriksson H, Bagge E, Båverud V, Fellström C, Jansson DS. Erysipelothrix rhusiopathiae contamination in the poultry house environment during erysipelas outbreaks in organic laying hen flocks. Avian Pathol 2014;43:231–237.
17. Pal N, Bender JS, Opriessnig T. Rapid detection and differentiation of Erysipelothrix spp. by a novel multiplex real-time PCR assay. J Appl Microbiol 2010;108:1083–1093.
18. Harada K, Uchiyama M, Hoshi T, Takahashi T. Comparison of three DNA extraction methods for detection of Erysipelothrix rhusiopathiae in chicken blood by polymerase chain reaction. J Vet Diagn Invest 2009;21:354–358.
19. Forde T, Biek R, Zadoks R, Workentine ML, De Buck J et al. Genomic analysis of the multi-host pathogen Erysipelothrix rhusiopathiae reveals extensive recombination as well as the existence of three generalist clades with wide geographic distribution. BMC Genomics 2016;17:461.
20. Shimoji Y. Pathogenicity of Erysipelothrix rhusiopathiae: virulence factors and protective immunity. Microbes Infect 2000;2:965–972.
21. Kaspers B, Lilleheij HS, Lilleheij EP. Chicken macrophages and thrombocytes share a common cell surface antigen defined by a monoclonal antibody. Vet Immunol Immunopathol 1993;36:333–346.
22. Dalgaard TS, Norup LR, Rubbenstroth D, Wattrang E, Juul-Madsen HR. Flow cytometric assessment of antigen-specific proliferation in peripheral chicken T cells by CFSE dilution. Vet Immunol Immunopathol 2010;138:85–94.
23. Wigley P. Immunity to bacterial infection in the chicken. Dev Comp Immunol 2013;41:413–417.
24. Genovese KJ, He H, Swaggerty CL, Kogut MH. The avian heterophil. Dev Comp Immunol 2013;41:334–340.
25. Ferdous F, Sasaki C, Bridges W, Burns M, Dunn H et al. Transcriptome profile of the chicken thrombocyte: new implications as an advanced immune effector cell. PLoS One 2016;11:e0163890.
26. Beuller E, Gelbart T, Kuhl W. Interference of heparin with the polymerase chain reaction. Biotechniques 1990;9:166.
27. Lam NY, Rainer TH, Chiu RW, Lo YM. EDTA is a better anticoagulant than heparin or citrate for delayed blood. Clin Chem 2004;50:256–257.
28. Nunes P, Demaurex N. The role of calcium signaling in phagocytosis. J Leukoc Biol 2010;88:57–68.
29. White-Owen C, Alexander JW, Sramkoski RM, Babcock GF. Rapid whole-blood microassay using flow cytometry for measuring neutrophil phagocytosis. J Clin Microbiol 1992;30:2071–2076.
30. Lennon JT, Muscarella ME, Placella SA, Lehmkuhl BK. How, when, and where Relic DNA affects microbial diversity. MBio 2018;9:e00637–00618.