DOI: 10.31073/onehealthjournal2026-III-02

PCR-based detection of *Erysipelothrix rhusiopathiae* using EvaGreen real-time PCR assay

Gerilovych A.[1, 2] (ORCID: 0000-0002-3280-4172), Korovin I.[1, 3], Vashchyk Ye.[3]
1 – PSI One Health Scientific and Research Institute, Kharkiv, Ukraine
2 – Institute for Problems of Cryobiology and Cryomedicine of NAS of Ukraine, Kharkiv, Ukraine
3 – National Scientific Center «Institute of Experimental and Clinical Veterinary Medicine», Kharkiv, Ukraine, email: korovin.vetbio@gmail.com

***Abstract**. The present study aimed to develop and evaluate an EvaGreen real-time PCR assay for rapid, sensitive, and specific detection of Erysipelothrix rhusiopathiae, the causative agent of swine erysipelas and an important zoonotic pathogen.*
Species-specific primers targeting the spaA gene of E. rhusiopathiae were used for amplification on the iTower real-time PCR platform (Jena Analytik, Germany). Reaction conditions were optimized using EvaGreen fluorescent chemistry. Analytical sensitivity was determined using ten-fold serial dilutions of purified genomic DNA, while specificity was evaluated against non-target bacterial species commonly associated with swine infections. Clinical samples obtained from pigs with suspected erysipelas, including tonsils, spleen, lymph nodes, heart valves, blood, and synovial fluid, were tested to assess diagnostic applicability.
The developed assay successfully detected E. rhusiopathiae DNA in all positive controls and field-positive samples. The limit of detection was established at 10 fg/µL. Standard curve analysis demonstrated excellent linearity (R^2 = 0.998) and high amplification efficiency (98.4%). No amplification was observed with non-target bacterial DNA, confirming high analytical specificity. All positive samples produced a single characteristic melting peak at 81.7 ± 0.3°C, indicating specific amplification. Among 48 field specimens, 19 samples (39.6%) tested positive, with the highest detection rates observed in tonsillar and splenic tissues. Intra- and inter-assay variation remained below 3%, confirming strong repeatability and reproducibility.
The EvaGreen real-time PCR assay is a rapid, reliable, and cost-effective molecular method for detection of E. rhusiopathiae. Its high sensitivity, specificity, and robust performance make it suitable for routine veterinary diagnostics, outbreak investigations, and epidemiological surveillance of swine erysipelas. The assay may serve as an effective alternative to probe-based real-time PCR systems, particularly in laboratories with limited resources.

Keywords: *Erysipelothrix rhusiopathiae*; swine erysipelas; EvaGreen; real-time PCR; molecular diagnostics; *spaA* gene.

Erysipelothrix rhusiopathiae is a Gram-positive, non-spore-forming, facultatively intracellular bacterium with worldwide distribution and substantial veterinary as well as zoonotic importance. It is recognized as the principal causative agent of erysipelas in swine, a disease characterized by acute septicemia, urticarial cutaneous lesions (“diamond skin disease”), chronic arthritis, and endocarditis, resulting in considerable economic losses to the pig industry through mortality, reduced productivity, and carcass condemnation (Wood, 1999; Opriessnig, 2011). In addition to swine, the pathogen has been isolated from poultry, sheep, fish, reptiles, and numerous wildlife species, demonstrating a broad host spectrum and ecological adaptability (Brooke, 1999, Zhao, 2023).
Beyond its veterinary relevance, *E. rhusiopathiae* is an established zoonotic pathogen capable of causing localized erysipeloid, generalized cutaneous infection, and occasionally severe systemic disease such as septicemia or infective endocarditis in humans. Occupational exposure remains the principal risk factor, particularly among farmers, butchers, veterinarians, fishermen, and abattoir workers who frequently handle infected animals or contaminated animal products (Wang, 2010; Milton, 2025; Rajkhowa, 2023). The One Health significance of the organism is therefore increasingly recognized, linking animal health, food safety, occupational medicine, and environmental hygiene.
The epidemiology of *E. rhusiopathiae* is further complicated by its ability to persist in asymptomatic carrier animals and survive for prolonged periods in the environment. Clinically healthy pigs may harbor the bacterium in tonsillar tissues and shed the organism intermittently, serving as a continuous source of infection within herds (Wood, 1999). Environmental persistence

in soil, slurry, and organic matter enhances indirect transmission and contributes to recurrent outbreaks, especially under conditions of inadequate sanitation, stress, overcrowding, or concurrent infections (Opriessnig, 2011).

Traditional laboratory diagnosis is based on bacterial isolation, microscopic examination, biochemical characterization, and serological methods. However, culture-based identification may be hindered by slow growth, overgrowth by contaminating flora, prior antimicrobial treatment, or low bacterial concentration in samples. These limitations can delay diagnosis and compromise timely disease control measures (Makino, 1994).

Molecular diagnostic approaches, particularly polymerase chain reaction (PCR), have markedly improved the detection of *E. rhusiopathiae* by providing rapid, sensitive, and highly specific identification directly from clinical specimens. PCR assays targeting genes such as 16S rRNA**,** spaA**,** and other species-specific markers are widely used for confirmation of infection, differentiation from related bacteria, and epidemiological investigations (Makino, 1994; Pal, 2010; Nishikawa, 2022). Real-time PCR platforms further enhance diagnostic efficiency by enabling quantitative detection, reduced contamination risk, and high-throughput screening suitable for veterinary surveillance programs.

Considering the continuing economic burden of erysipelas, the zoonotic potential of the pathogen, and the necessity for rapid outbreak response, PCR-based methods have become indispensable tools in modern veterinary diagnostics. Therefore, the application and optimization of PCR for detection of *E. rhusiopathiae* remain highly relevant for disease monitoring, herd health management, and integrated One Health biosecurity strategies.

Therefore, the **aim of this study** was to develop and validate a real-time PCR assay based on EvaGreen chemistry using the iTOWER platform (Jena Analytik) for the detection of *E. rhusiopathiae* DNA targeting the *spaA* gene. The proposed method is intended to improve diagnostic efficiency and support integrated disease monitoring within the One Health framework.

Materials and Methods. The study was conducted to develop and evaluate a real-time PCR assay for the detection of *Erysipelothrix rhusiopathiae* using EvaGreen fluorescent chemistry. Reference strains of *E. rhusiopathiae* obtained from a certified microbial collection were used as positive controls. To assess analytical specificity, non-target bacterial species commonly associated with swine infections or capable of causing similar clinical manifestations were included, such as *Streptococcus suis*, *Staphylococcus aureus*, *Escherichia coli*, *Pasteurella multocida*, and *Trueperella pyogenes*.

Clinical samples were collected from pigs showing signs consistent with swine erysipelas, including fever, skin lesions, lameness, arthritis, and sudden death. Specimens included tonsils, spleen, liver, heart valves, lymph nodes, synovial fluid, and blood. Tissue samples were collected aseptically during necropsy and transported to the laboratory under refrigerated conditions (4°C) for further processing.

Sample preparation and DNA extraction. Approximately 25 mg of tissue was homogenized in sterile phosphate-buffered saline using disposable homogenizers. Blood and synovial fluid samples were vortexed thoroughly before extraction. Genomic DNA was extracted using a commercial silica membrane-based purification kit according to the manufacturer’s instructions. DNA was eluted in 100 µL of nuclease-free water and stored at −20°C until analysis.

DNA concentration and purity were measured spectrophotometrically at 260/280 nm. Only samples with acceptable purity ratios (1.7–2.0) were included for PCR testing.

Primer design. Species-specific primers were selected from conserved regions of the *spaA* gene encoding surface protective antigen A, commonly associated with virulent strains of *E. rhusiopathiae*. Primer sequences were analyzed for specificity using nucleotide database alignment tools and synthesized commercially.

Table 1

Primer set for *E. rhusiopathiae* target gene sequence amplifcation

Primer	Sequence (5′–3′)	Target Gene
ERH-F	AGT TTA CGC TGA TGA AGG TG	*spaA*
ERH-R	TCA ATC CTT GCA TTT CCA GC	*spaA*

Expected amplicon size by using of selected primers was 128 bp.

Real-time PCR assay. Amplification was performed using an iTower real-time PCR system (Jena Analytik, Germany) in a final reaction volume of 20 µL containing:

- 10 µL 2× EvaGreen qPCR Master Mix
- 0.4 µM forward primer
- 0.4 µM reverse primer
- 2 µL template DNA
- nuclease-free water to final volume

Thermal cycling conditions were as follows:

Initial denaturation: 95°C for 5 min, and 40 cycles of denaturation: 95°C for 15 s, annealing: 58°C for 20 s and extension: 72°C for 20 s

Fluorescence acquisition was performed at the end of each extension step.

Melting curve analysis. Following amplification, melt curve analysis was carried out from 65°C to 95°C with increments of 0.2°C every 5 s to verify product specificity. A single sharp melting peak was interpreted as specific amplification of the target fragment.

Analytical sensitivity. To determine the limit of detection (LOD), ten-fold serial dilutions of purified *E. rhusiopathiae* DNA were prepared from 10 ng/µL to 1 fg/µL. Each dilution was tested in triplicate. The lowest concentration consistently detected in all replicates was considered the analytical detection limit.

Analytical specificity. DNA extracted from non-target bacterial species was tested under identical reaction conditions to evaluate cross-reactivity. Absence of amplification or non-specific melt peaks was interpreted as assay specificity.

Standard curve and PCR efficiency. A standard curve was generated using serial DNA dilutions by plotting threshold cycle (Ct) values against the logarithm of DNA concentration. Amplification efficiency (E) was calculated using the formula:

$$E = (10^{-1/slope} - 1) \times 100\%$$

The correlation coefficient (R^2) was used to assess linearity of amplification.

Repeatability and reproducibility. Intra-assay repeatability was determined by testing three DNA concentrations in triplicate within a single run. Inter-assay reproducibility was evaluated by repeating experiments on three separate days. Mean Ct values, standard deviations, and coefficients of variation were calculated.

Data interpretation. Samples with Ct values ≤35 and a specific melting temperature corresponding to the positive control were considered positive. Samples with Ct values >35 were retested. No-template controls were included in each run to monitor contamination.

Statistical analysis. Descriptive statistics, regression analysis, and calculation of mean Ct values were performed using Microsoft Excel and statistical software. Differences were considered significant at $p < 0.05$ where applicable.

Results. *Performance of the EvaGreen real-Time PCR assay.* The developed EvaGreen real-time PCR assay successfully detected *Erysipelothrix rhusiopathiae* DNA in all positive control samples and in clinically suspected field specimens containing the target organism. Amplification curves demonstrated typical sigmoidal kinetics with clear fluorescence separation from the baseline and no signal in no-template controls.

The assay generated reproducible amplification over a wide dynamic range of DNA concentrations. Positive reactions were consistently observed from 10 ng/µL to the lowest detectable dilution, indicating high analytical sensitivity.

Analytical sensitivity and standard curve analysis. Ten-fold serial dilutions of purified *E. rhusiopathiae* DNA were used to determine the analytical sensitivity and amplification efficiency. The assay consistently detected target DNA down to 10 fg/µL, which was established as the limit of detection (LOD). At concentrations below this level, amplification became inconsistent and Ct values were highly variable.

A linear relationship was observed between Ct values and logarithmic DNA concentration across six dilution points. Standard curve analysis demonstrated excellent linearity with a correlation coefficient of $R^2 = 0.998$, indicating reliable quantitative performance. The slope of the standard curve was −3.36, corresponding to a PCR efficiency of 98,4 % (table 2).

$$E = (10^{-1/(-3.36)} - 1) \times 100\% = 98.4\%$$

These values indicate optimal amplification characteristics for diagnostic real-time PCR.

Table 2

Analytical sensitivity and standard curve parameters

Parameter	Value
Dynamic range	10 ng/μL – 10 fg/μL
Limit of detection (LOD)	10 fg/μL
Slope	−3.36
Efficiency (E%)	98.4%
Correlation coefficient (R^2)	0.998

Specificity of the assay. No amplification signals were observed from DNA extracted from non-target bacterial species including *Streptococcus suis*, *Staphylococcus aureus*, *Escherichia coli*, *Pasteurella multocida*, and *Trueperella pyogenes*. These findings confirmed high analytical specificity of the primer pair and absence of cross-reactivity under optimized reaction conditions.

Melting curve analysis. All positive *E. rhusiopathiae* samples produced a single sharp melting peak at 81.7 ± 0.3°C, confirming specific amplification of the intended amplicon. No secondary peaks, primer-dimer signals, or abnormal melting profiles were detected.

*Detection in clinical samples.*A total of 48 field samples from pigs with suspected erysipelas were examined. Of these, 19 samples (39.6%) were positive by EvaGreen real-time PCR. Positive detections were most frequent in tonsils, spleen, lymph nodes, and heart valve tissues. Blood samples showed lower positivity rates, particularly in animals sampled after antimicrobial treatment (table 3).

Table 3.

Detection of *E. rhusiopathiae* in field specimens

Sample type	Number tested	Positive	Positivity (%)
Tonsils	12	7	58.3
Spleen	8	4	50.0
Lymph nodes	7	3	42.9
Heart valves	5	2	40.0
Blood	10	2	20.0
Synovial fluid	6	1	16.7
Total	**48**	**19**	**39.6**

Repeatability and reproducibility. The assay showed strong repeatability with intra-assay coefficient of variation (CV) values ranging from 0.8% to 1.9%. Inter-assay reproducibility across three independent runs demonstrated CV values between 1.4% and 2.7%, indicating robust assay stability.

Overall diagnostic utility. The EvaGreen real-time PCR assay provided rapid detection of E. rhusiopathiae within approximately 70 minutes, including amplification and melt curve analysis. The combination of high sensitivity, excellent specificity, and reproducible performance supports its suitability for routine veterinary diagnostics, outbreak investigations, and surveillance of swine erysipelas.

Conclusions. The developed EvaGreen real-time PCR assay proved to be a rapid, sensitive, and specific method for the detection of *Erysipelothrix rhusiopathiae*. The assay reliably identified target DNA over a broad dynamic range and demonstrated excellent analytical performance, including a low detection limit, high amplification efficiency, and strong linearity of the standard curve.

Melting curve analysis confirmed the specificity of amplification by producing a single characteristic melting peak for positive samples, while no cross-reactivity was observed with non-target bacterial species commonly associated with swine infections. These findings indicate that the selected primers and optimized reaction conditions are suitable for accurate laboratory diagnosis.

Application of the assay to clinical field samples enabled efficient identification of infected animals, with the highest detection rates observed in tonsils and internal organs, supporting the known role

of carrier pigs and systemic dissemination during disease. The method also showed high repeatability and reproducibility, confirming its robustness for routine diagnostic use.

Overall, the EvaGreen real-time PCR assay represents a practical and cost-effective molecular tool for veterinary laboratories, particularly where probe-based systems are less accessible. Its implementation can significantly improve early diagnosis, outbreak response, epidemiological surveillance, and biosecurity management of swine erysipelas. Further validation using larger sample sets and comparison with bacteriological culture methods would strengthen its application in national monitoring programs and integrated One Health disease control strategies.

REFERENCES

1. Brooke C. J., Riley T. V. (1999). *Erysipelothrix rhusiopathiae*: Bacteriology, epidemiology and clinical manifestations of an occupational pathogen. *Journal of Medical Microbiology, 48*(9), 789–799. https://doi.org/10.1099/00222615-48-9-789
2. Gerilovych A. P. (2011). *Eksperymentalne i teoretychne obgruntuvannia ta rozrobka zasobiv epizootolohichnoho monitorynhu, diahnostyky virusnykh khvorob tvaryn ta molekuliarno-henetychnoho typuvannia yikh zbudnykiv (ortomikso-, paramikso-, herpes-, tsyrko- ta pestyvirusna infektsii)* [Experimental and theoretical substantiation and development of means of epizootological monitoring, diagnostics of viral diseases of animals and molecular genetic typing of their pathogens (orthomyxo-, paramyxo-, herpes-, circo- and pestivirus infections)] (Doctoral dissertation summary, National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine"). Kharkiv, Ukraine.
3. Makino S., Okada Y., Maruyama T., Ishikawa K., Takahashi T., Nakamura M., Ezaki T., Morita H. (1994). Direct and rapid detection of *Erysipelothrix rhusiopathiae* DNA in animals by PCR. *Journal of Clinical Microbiology, 32*(6), 1526–1531.
4. Milton A. A. P., Khan S., Srinivas K., Basar T., Hussain Z., Priya G. B., Saminathan M., Puii L. H., Ghatak S., Das S. (2025). Novel multiplex PCR approach for the detection of *Erysipelothrix rhusiopathiae*, *Streptococcus suis*, and *Staphylococcus hyicus* in swine. *Frontiers in veterinary science*, *12*, 1605316. https://doi.org/10.3389/fvets.2025.1605316
5. Nishikawa S., Shiraiwa K., Shimoji Y. (2022). A PCR assay to specifically detect serovar 1a strains of Erysipelothrix rhusiopathiae and differentiate them from serovar 2 strains possessing an intact ERH_1440 gene. *The Journal of veterinary medical science*, *84*(1), 90–93. https://doi.org/10.1292/jvms.21-0528
6. Opriessnig T., Wood R. L., Eamens G. J. (2011). *Erysipelas*. In B. E. Straw J. J. Zimmerman, S. D'Allaire D. J. Taylor (Eds.), *Diseases of Swine* (10th ed., pp. 750–759). Wiley-Blackwell.
7. Pal N., Bender J. S., Opriessnig T. (2010). Rapid detection and differentiation of *Erysipelothrix* species by multiplex real-time PCR. *Journal of Applied Microbiology, 108*(1), 285–294. https://doi.org/10.1111/j.1365-2672.2009.04400.x
8. Rajkhowa S., Sonowal J., Borthakur U., Pegu S. R., Deb R., Das P. J., Sengar G. S., Gupta V. K. (2023). Meta-Analysis of the Prevalence of Porcine Zoonotic Bacterial Pathogens in India: A 13-Year (2010-2023) Study. *Pathogens (Basel, Switzerland)*, *12*(10), 1266. https://doi.org/10.3390/pathogens12101266
9. Wang Q., Chang B. J., Riley T. V. (2010). *Erysipelothrix rhusiopathiae*. *Veterinary Microbiology, 140*(3–4), 405–417. https://doi.org/10.1016/j.vetmic.2009.08.012
10. World Organisation for Animal Health. (2021). *Manual of diagnostic tests and vaccines for terrestrial animals* (8th ed.). https://www.woah.org/en/what-we-do/standards/codes-and-manuals/#chapter/?rid=298&volume_no=3&ismanual=true&language=102&standard_type=6&animal_type=7
11. Zhao L., Wen X. H., Jia C. L., Zhou X. R., Luo S. J., Lv D. H., Zhai Q. (2023). Development of a multiplex qRT-PCR assay for detection of classical swine fever virus, African swine fever virus, and *Erysipelothrix rhusiopathiae*. *Frontiers in veterinary science*, *10*, 1183360. https://doi.org/10.3389/fvets.2023.1183360.

Виявлення *Erysipelothrix rhusiopathiae* методом ПЛР у реальному часі із використанням EvaGreen

Герілович А.[1, 2] (ORCID: 0000-0002-3280-4172), Коровін І.[1, 3], Ващик Є. [3]
1 – ПНУ «Науково-дослідний інститут єдиного здоров'я», м. Харків, Україна
2 – Інститут проблем кріобіології та кріомедицини НАН України, м. Харків, Україна
3 – Національний науковий центр «Інститут експериментальної і клінічної ветеринарної медицини», м. Харків, Україна, електронна пошта: korovin.vetbio@gmail.com

Резюме. *Метою даного дослідження було розроблення та оцінка методу ПЛР у реальному часі з використанням EvaGreen для швидкого, чутливого та специфічного виявлення Erysipelothrix rhusiopathiae, збудника бешихи свиней та важливого зоонозного патогену.*
Для ампліфікації на платформі ПЛР у реальному часі iTower (Jena Analytik, Німеччина) використовували видоспецифічні праймери, спрямовані на ген spaA E. rhusiopathiae. Умови реакції були оптимізовані з використанням флуоресцентного барвника EvaGreen. Аналітичну чутливість визначали за допомогою десятикратних послідовних розведень очищеної геномної ДНК, тоді як специфічність оцінювали щодо нецільових видів бактерій, які зазвичай зумовлюють інфекції у свиней. Клінічні зразки, отримані від свиней з підозрою на бешиху, включаючи мигдалики, селезінку, лімфатичні вузли, серцеві клапани, кров та синовіальну рідину, були протестовані для оцінки діагностичної застосовності.
Розроблений тест успішно виявив ДНК E. rhusiopathiae у всіх позитивних контролях та польових позитивних зразках. Межа виявлення була встановлена на рівні 10 фг/мкл. Аналіз стандартної кривої продемонстрував чудову лінійність (R^2 = 0,998) та високу ефективність ампліфікації (98,4%). Ампліфікації не спостерігалося з ДНК нецільових бактерій, що підтвердило високу аналітичну специфічність. Усі позитивні зразки дали один характерний пік плавлення при 81,7 ± 0,3 °C, що вказує на специфічну ампліфікацію. Серед 48 польових зразків 19 (39,6 %) дали позитивний результат, причому найвищі показники виявлення спостерігалися у тканинах мигдаликів та селезінки. Внутрішньо- та міжтестова варіація залишалася нижче 3 %, що підтверджує високу повторюваність та відтворюваність.
Аналіз EvaGreen у режимі реального часу є швидким, надійним та економічно ефективним молекулярним методом виявлення E. rhusiopathiae. Його висока чутливість, специфічність та надійність роблять його придатним для рутинної ветеринарної діагностики, розслідування спалахів та епідеміологічного нагляду за еризепелою свиней. Цей аналіз може слугувати ефективною альтернативою системам ПЛР у режимі реального часу на основі зондів, особливо в лабораторіях з обмеженими ресурсами.
Ключові слова: *Erysipelothrix rhusiopathiae*; бешиха свиней; EvaGreen; ПЛР у реальному часі; молекулярна діагностика; ген spaA.
DOI: 10.31073/onehealthjournal2026-III-02