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DETECTION OF ANAPLASMA INFECTION IN ASYMPTOMATIC COWS

Deteksi Anaplasma pada sapi yang tidak menunjukkan gejala klinis

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Abstract

The diagnosis of *Anaplasma* is typically carried out through morphological identification based on the presence of inclusion bodies located at the margins of erythrocytes. Microscopic examination of blood smears stained with Wright's–Giemsa is generally suitable for detecting acute anaplasmosis in clinically suspected animals. This study aimed to compare the diagnostic accuracy of microscopic examination and conventional polymerase chain reaction (PCR) for detecting *A. marginale* in asymptomatic (carrier) cattle. A total of 385 blood samples were collected from cows without clinical symptoms. The sensitivity and specificity of microscopic detection were evaluated against conventional PCR results. The sensitivity and specificity of microscopic results were compared with *A. marginale* by conventional PCR. The results revealed 3.40% and 12.73% positive animals by microscopy and conventional PCR with significant differences (P=0.03). The value of Kappa between microscopic examination and conventional PCR has indicated a fair level of agreement (0.32). Microscopic examination showed 6.10% sensitivity and 97.40% specificity compared to conventional PCR's 100% sensitivity and specificity. These results indicate that conventional PCR is a more accurate and reliable method for detecting *A. marginale* in asymptomatic cattle. The limitations of microscopy, particularly in carrier animals, include the very low number of infected erythrocytes, difficulty in identifying well-stained *Anaplasma* organisms, and challenges in distinguishing *A. marginale* from *A. centrale*.

Keywords: *Anaplasma,* microscopy, PCR, sensitivity, specificity

Abstrak

Diagnosis *Anaplasma* biasanya dilakukan melalui identifikasi morfologi berdasarkan keberadaan badan inklusi yang terletak di tepi eritrosit. Pemeriksaan mikroskopis terhadap

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apusan darah yang diwarnai dengan Wright–Giemsa umumnya cocok untuk mendeteksi anaplasmosis akut pada hewan yang diduga secara klinis. Penelitian ini bertujuan untuk membandingkan akurasi diagnostik dari pemeriksaan mikroskopis dan *polymerase chain reaction* (PCR) konvensional untuk mendeteksi *A. marginale* pada sapi tanpa gejala (pembawa/*carrier*). Sebanyak 385 sampel darah dikumpulkan dari sapi yang tidak menunjukkan gejala klinis. Sensitivitas dan spesifisitas deteksi mikroskopis dievaluasi dengan membandingkannya terhadap hasil PCR konvensional. Hasil penelitian mengungkapkan 3,40% dan 12,73% hewan positif masing-masing melalui mikroskopis dan PCR konvensional, dengan perbedaan yang signifikan (P=0,03). Nilai Kappa antara pemeriksaan mikroskopis dan PCR konvensional menunjukkan tingkat kesepakatan yang cukup (0,32). Pemeriksaan mikroskopis menunjukkan sensitivitas 6,10% dan spesifisitas 97,40% bila dibandingkan dengan PCR konvensional yang memiliki sensitivitas dan spesifisitas 100%. Hasil ini menunjukkan bahwa PCR konvensional merupakan metode yang lebih akurat dan andal untuk mendeteksi *A. marginale* pada sapi tanpa gejala. Keterbatasan mikroskopis, khususnya pada hewan pembawa, mencakup jumlah eritrosit yang terinfeksi yang sangat rendah, kesulitan dalam mengidentifikasi organisme *Anaplasma* yang terwarnai dengan baik, serta tantangan dalam membedakan *A. marginale* dari *A. centrale*.

Kata kunci: *Anaplasma*, mikroskopis, PCR, sensitifitas, spesifisitas

INTRODUCTION

Bovine anaplasmosis is a hemoparasitic disease in cattle caused by bacteria of the genus *Anaplasma*. The primary species affecting cattle and other animals include *Anaplasma marginale*, *A. centrale*, *A. bovis*, *A. phagocytophilum*, *A. ovis*, and *A. platys* (Belkahia et al., 2015; Ben Said et al., 2018). Among these, *A. marginale* is the principal pathogen responsible for bovine anaplasmosis and is associated with significant economic losses in the dairy industry (Yang et al., 2017).

Diagnosis of *A. marginale* is commonly performed through morphological identification of inclusion bodies located at the periphery of erythrocytes. Microscopic examination of blood smears stained with Wright–Giemsa is effective for diagnosing acute cases of anaplasmosis in clinically suspected animals (Noaman & Shayan, 2010; Wahba, 2017). However, this method is not reliable for detecting asymptomatic or carrier animals.

After transmission, *Anaplasma marginale* invades and replicates within mature red blood cells. During the acute phase of anaplasmosis, rickettsemia levels can exceed 10^9 infected erythrocytes per millilitre, leading to severe clinical manifestations such as anaemia, weight loss, abortion, and even death (Bisen et al., 2021). Animals that recover from acute infection remain persistently infected, experiencing recurrent cycles of rickettsemia ranging from approximately $10^{2.5}$ to 10^7 infected erythrocytes per millilitre (Bisen et al., 2021; Parodi et al., 2022). These persistently infected cattle act as long-term reservoirs, facilitating disease transmission within herds. Therefore, identifying persistently infected animals is crucial to preventing the spread of infection and controlling the movement of infected cattle to and from disease-free areas.

The conventional identification of *Anaplasma marginale* through Giemsa-stained blood smear examination is laborious, time-consuming, and has limited diagnostic accuracy. This method relies on detecting inclusion bodies at the erythrocyte margins and is only effective when parasitaemia exceeds 10^6 infected cells per millilitres, making it suitable for acute cases but unreliable for identifying carrier or pre-symptomatic animals (Selim et al., 2021). Moreover, the low number of infected erythrocytes in carriers, difficulty in distinguishing *A. marginale*

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from *A. centrale*, and the potential confusion with structures like Heinz bodies or staining artifacts further limit its diagnostic value (Sharma et al., 2014).

The initial bodies of *A. marginale* express several outer membrane proteins that stimulate the host's immune system to produce antibodies. These proteins, known as major surface proteins (MSPs), include MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4, and MSP-5. Among them, MSP-1a, MSP-4, and MSP-5 are conserved during replication and show no variation among isolates (De la Fuente et al., 2002). MSP-1a and MSP-4 are often used to assess the genetic diversity of *Anaplasma* species (De la Fuente et al., 2003; Kocan et al., 2010). Molecular techniques with high sensitivity and specificity have been developed for the detection of *Anaplasma marginale* DNA, among which the polymerase chain reaction (PCR) assay is regarded as the "gold standard" for identifying persistently infected cattle.

MATERIALS AND METHODS

Research Design and Ethics

The Institutional Animal Care and Use Committee of Khon Kaen University approved animal use in the study, with the recorded number IACUC-KKU-127/64 and reference number 660201.2.11/656 (122). The current study was conducted on smallholder dairy cattle farms in five districts of Khon Kaen between July 2020 and October 2021. Standard techniques were followed during the collection of blood samples.

Samples

A total of 385 apparently healthy lactating dairy cattle from 40 farms, regardless of age, and health status, were included. Approximately 5mL of blood sample was collected from the caudal vein of each cattle and transferred to a sterile K2 EDTA Vacutainer® tube (Nipro, Shanghai Co., Ltd). Thin blood smears were prepared for Peripheral Blood Smear Examination (PBSE). The slides were stained with Wright-Giemsa-staining (Fosgate et al., 2010). Genomic DNA was extracted from 200 µL of blood using a commercial spin column-based extraction kit (GF-1 Blood DNA Extraction Kit, Vivantis Technologies, Malaysia) following the manufacturer's protocol.

Molecular Examination and Data Analysis

For molecular analysis, the target fragments were amplified by using the forward primer 'msp43: 5'-CCG GAT CCT TAG CTG AAC AGG AAT CTT GC-3' and reverse primer 'msp45: 5'-GGG AGC TCC TAT GAA TTA CAG AGA ATT GTT TAC-3' to amplify 849 bp target (de la Fuente et al., 2002). The positive control of *A. marginale* was kindly provided by the National Institute of Animal Health, Department of Livestock Development, Bangkok. The PCR products were checked for amplification by electrophoresis on a 1.5% agarose gel and visualized using a gel documentation system. The results of the PCR assay were compared with that of the Wright's-Giemsa-stained blood smear examination.

RESULT AND DISCUSSION

Results

In the present study, Wright's-Giemsa-stained thin blood smear examination of apparently healthy cattle revealed 3.40% (13/385) positive for the inclusion bodies of *A. marginale* and 12.73% (49/385) were positive using PCR assay. The value of Kappa between microscopic examination and single PCR assay has indicated a fair level of agreement (0.32). The comparison results compared with PCR assay, from 13 positive samples by microscopic examination, three samples were true positive, and ten were false positive for *A. marginale*.

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The microscopic examination showed 6.10% sensitivity and 97.40% specificity compared to the PCR assay (Table 1).

Traditionally, microscopic examination of Wright's-Giemsa-stained blood smears has been used to diagnose acute anaplasmosis as well as to detect carrier animals; however, this approach faces significant limitations. Serological tests have also been developed for diagnosing anaplasmosis, but due to cross-reactivity issues, they are not reliable for distinguishing Anaplasma infections from other similar diseases. The findings indicated that conventional microscopic examination of blood smears is inadequate for detecting low bacteraemia levels in carrier cattle. Moreover, Anaplasma-like structures observed in erythrocytes are often difficult to distinguish from Heinz bodies, Howell–Jolly bodies, or staining artifacts. Given the extremely low proportion of infected erythrocytes (approximately 0.01%–0.001%) in carrier animals, identifying Anaplasma organisms through routine Giemsa staining, is highly challenging.

Discussion

Anaplasmosis is one of serious health problems that cause reduced animal productivity and economic losses. The most commonly used method for diagnosing Anaplasma infection in cattle is a microscopic examination of Giemsa-stained or Wright's-Giemsa-stained blood smears. However, carrier cattle have a low level of infected erythrocytes and difficulty distinguishing between Anaplasma organisms and other structures like Heinz-bodies, Howell-Jolly bodies, or staining artefacts (Al-Ethafa et al., 2019); this method is not recommended for the characterization of this pathogen.

To enhance the sensitivity and specificity of the diagnosis, the PCR was performed using msp4 specific primers. In the present study, out of 385 blood samples, 49 samples (12.73%) were positive for *A. marginale*, revealing an expected amplicon of 849 bp (Figure 1.) by PCR analysis. In contrast, Wright's-Giemsa-staining analysis identified *A. marginale*-like structures in only 13 blood samples (3.40%). Similarly, in a study conducted in India, demonstrated that the prevalence of *A. marginale* by microscopic examination and PCR assay were 24% and 50% (Singh et al., 2012, 2014), respectively. These findings indicate that PCR-based assay is more sensitive and specific than a microscopic method to diagnose *A. marginale* infections. In Thailand, bovine anaplasmosis has been reported since 1986 in beef cattle and during 2001 to 2017 in water buffaloes, for examples, Nan, Nakhon Sawan, Ayutthaya, Roi Et, Ubon Ratchathani, Satun, Surin, and Nakhon Si Thammarat, ranging from 0.03 to 65.2% (Jirapattharasate et al., 2017; Saetiew et al., 2020). The current study results have revealed that *A. marginale* infection of dairy cattle was similar to 12.0% in beef cattle using PCR assay in Khon Kaen Province (Jirapattharasate et al., 2016).

Accurate microscopic detection of Anaplasma requires high bacteraemia levels, well-prepared smears, proper staining, and skilled personnel, although the technique remains inexpensive and simple to perform. Despite its limitations, microscopy is still a practical method for routine diagnosis of acute anaplasmosis in laboratories. However, for identifying carrier animals with low bacteremia, examining more microscopic fields is more effective than less of 10 fields. Overall, conventional PCR proved to be a reliable and sensitive method for detecting cattle persistently infected with Anaplasmosis.

CONCLUSION AND SUGGESTION

Conclusion

Microscopy of blood smears, especially from asymptomatic cows, is accompanied by several problems due to the meager amount of infected erythrocytes in carrier animals, the limited

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detection of good stained Anaplasma organisms, and the difficulty to distinguish between *A. marginale* and *A. centrale*. The present study identified bovine anaplasmosis caused by Anaplasmosis. Consequently, the PCR assay diagnoses the incidence of *A. marginale* infection even in asymptomatic cattle with low parasitemia.

Suggestions

Routine surveillance programs should incorporate molecular diagnostics, such as conventional or real-time PCR, to detect *A. marginale* in asymptomatic carriers. Microscopic examination may remain useful for rapid preliminary screening in field settings but should be confirmed by molecular methods.

Future research should focus on developing cost-effective and field-deployable molecular assays, such as loop-mediated isothermal amplification (LAMP) or portable PCR platforms. Sequencing of msp4 or other genetic markers is also recommended to explore the genetic diversity and potential strain variation of *A. marginale* in Southeast of Asia.

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Table

Table 1. The sensitivity and specificity of microscopic examination compared to 100% sensitivity and specificity of PCR assay on detection of *A. marginale*

Method	No. of positive sample	% of positive sample	Sensitivity (%)[a]	Specificity (%)[b]
PCR	49/385	12.73	100	100
Microscopic	13/385	3.40	6.10	97.40

For microscopic examination, the number of true positives, false positives, true negatives, and false negatives was 3, 10, 46, and 326, respectively. [a]Calculated as follows: [number of true positives/(number of true positives + number of false negatives)] × 100. [b]Calculated as follows: [number of true negatives/(number of true negatives + number of false positives)]× 100. (Noaman & Shayan, 2010)

Figure.

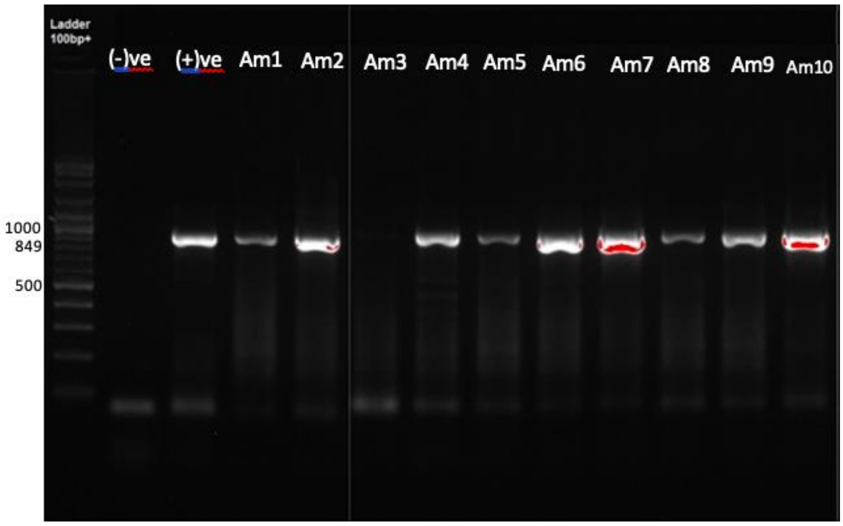

Figure 1. Single PCR assay of amplification products based on the *msp4* gene of *A.marginale*. The expected size (849 bp) is indicated. Positive samples = Am1, Am2, Am3, Am4, Am5, Am6, Am7, Am8, Am9, Am10. (-)ve = negative control. (+)ve = positive control. Ladder = 100-bp DNA Ladderplus (Vivantis Technologies, Malaysia).

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