Abstract
Introduction: Livestock is the potential source of blood feed for Anopheles mosquitoes. Infectious Anopheles bites enter h-Plasmodium sporozoites in the body of livestock. In the erythrocytic phase, the parasite produces several enzymes, one of which is parasite lactate dehydrogenase (pLDH). The parasite needs this enzyme to help the glycolysis process of pyruvate into lactic acid.

Methods: In this cross-sectional study, the presence of pLDH in blood specimens of Etawa crossbreed goats was explored. Samples of the stored biological material in the form of dried blood in Whatman™ 1001 125 filter paper included 97 specimens. pLDH was detected using the PCR method.

Results: One sample previously reported to contain P. falciparum trophozoites under microscopic examination showed positive results in pLDH testing.

Conclusion: The parasite of P. falciparum was found capable of living up to the erythrocytic phase and forming PfLDH in the Etawa crossbreeds.

Keywords: PfLDH, Etawa crossbred goats, Malaria, PCR-methods

Introduction
Domestic goats have the potential to be sources of blood for zoophilic Anopheles mosquitoes. The bite of a malaria vector introduces human-Plasmodium (h-Plasmodium) sporozoites in the target animal (1,2). When the parasite’s life in the host reaches the erythrocytic phase, several metabolic products are produced, including histidine-rich protein (HRP), parasite lactate dehydrogenase (pLDH), and aldolase (3–5). All these three are markers of the presence of h-Plasmodium in the host’s body. Although it is not the gold standard for malaria testing (6), the appearance of these markers still provides important information regarding the transmission that occurs.

The pLDH enzyme is produced by the h-Plasmodium parasite when it enters the erythrocytic phase (7). The enzyme formation is required to support the life of the parasite itself through the process of glycolysis which converts pyruvate to lactic acid (8). The pLDH emerges along with the process of h-Plasmodium infection that occurs in the host. The pLDH enzyme in human cases lasts relatively long in the blood, and it may even be detected several days after the patient receives treatment (9). Information on h-Plasmodium life in domestic animals has not been widely disclosed.

Transmission of h-Plasmodium to vertebrate hosts cannot be separated from the role of the Anopheles spp mosquito as a malaria vector (10-12). Some vectors are zoophilic, meaning that they prefer animal blood to human’s (13). It is the zoophilic nature to search for blood feed sources in animals. The presence of a group of livestock in one cage can provide more inviting environment for zoophilic Anopheles (14). The search for blood feed sources among female mosquitoes is of a vital necessity to sustain life (15). Inevitable foraging activities expose the domestic animals to the attacks by female zoophilic Anopheles (13,16).

Many studies have failed to reveal the presence of h-Plasmodium in animals and livestock. Identifying h-Plasmodium in a group of domestic cattle in Sumba and Fakfak, however, has confirmed the fact that this parasite can live in the livestock (17). A similar finding in goats was reported in Jatirejo by microscopic examination of
thick blood smears (18), although some practitioners still doubt it. The presence of *h-Plasmodium* in domestic livestock is a new warning signal for those living in, especially, malaria-endemic and pre-elimination areas since it indicates that the parasite is also capable of breeding in non-human vertebrate hosts.

Malaria pre-elimination areas must strive to maintain zero local cases for three years to achieve malaria elimination status (19,20). Many factors must be taken care of for the elimination program to be fruitful. Promotive, preventive, and curative efforts must be undertaken separately and/or in synergy (21). Patient management alone is not sufficient to control the spread of malaria (22). It requires best efforts to control the vector of transmission and suppress the potential for wider transmission (23,24).

If *h-Plasmodium* can live in the livestock, it means that the malaria control program is not enough to manage the sufferers as well as their vectors and, therefore, the program must also include the management of livestock rearing. As the result, detecting the presence and survival strategies of *h-Plasmodium* in domestic livestock assumes an added importance. This study, therefore, aimed to detect the presence of pLDH in stored biological materials that previously had been reported to contain the *h-Plasmodium* parasite.

**Materials and Methods**

**Materials**
The subject of the study was biological a material stored in a dried blood spot in Whatman™ 1001 125 filter paper. The previously reported samples contained *h-Plasmodium* Human Plasmodium-like was reported previously as trophozoite form on thick blood smears(25). This biological material, having been stored in the Public Health Laboratory of the Universitas Muhammadiyah Semarang, was the residue from previous research. As for the information about the specimens, the blood samples were obtained from the Itawa crossbreed goats aged at least three months and kept in Jatirejo village. The total number of the biological materials was 97.

**Study Design**
An observational study was conducted to explore the emergence of pLDH in blood of goats from the malaria pre-elimination areas by performing laboratory testing.

**Polymerase Chain Reactions Testing**

**DNA Isolation**
Spots of dried blood on filter paper were cut with a sterile perforator and put into a 1.5 mL microtube containing 200 µL of InstaGene Matrix (Bio-Rad Laboratories, USA). Then it was incubated at 56°C for 30 minutes. The sample was vortexed for 10 seconds at 15 and 30 minutes during incubation. The specimen was heated at 100°C for 8 minutes, and centrifuged at 15000 g for 2 minutes. The supernatant was taken carefully and stored at minus 20°C as a result of DNA isolation.

**DNA Amplification**
Primer (Macrogen Humanizing Genomics) was diluted according to the directions in reagent kit. The base sequences for *Plasmodium falciparum* were 5’ATGGGACAAAAAGCAAA3’ (Forward) and 5’TTCAGCTATTGCCTTCATTCTC3’ (Reverse), while those for *Plasmodium vivax* were 5’CCATGGCTATGACGCGAAAAACTTTTTG3’ (Forward) and 5’TCGAGAATGAGCCGCTTTCATCCC3’ (Reverse) (26). DNA template (2.5 µL), forward–reverse primer (1 µL each), 2x Taq plus Tiangen KT205 master-mix (5 µL), and nuclease-free water (3 µL) were added to the content of PCR tube. Amplification was done in Bio-Rad Thermal Cycler-100. Pre-denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 53.5°C for 30 seconds, extension at 68°C for 1 minute, final extension at 68°C for 5 minutes, and cooling at 4°C with 40 cycles of repetition were all performed.

**Electrophoresis**
Agarose weighing 1 g was added to 100 mL of 1% TBE solution, mixed well, and heated in the oven for 1-2 minutes. While the mixture was hot, 25 µL of GelRed nucleic acid stain (Biotium Inc, USA) was added to it and homogenized. Agarose solution was poured into a mold with a well of some samples tested. The comb was removed from the agarose after it has cooled. The agarose gel was placed on Consort-Electrophoresis and filled with 1% TBE solution. Ten microliter markers of 100 base pairs were inserted into the first well from the left side, followed by negative control and PCR test samples. Electrophoresis was processed at 100 V for 60 minutes. It was difficult to get positive controls; therefore, the negative control was used. The negative control was a parasite-free sample on microscopic examination.

**Result Reading**
Agarose gel was installed in the Mini Illuminator. The target DNA appeared in orange colour under UV. The photo documentation was taken from the electrophoresis results by a mini illuminator. The concentration of the sample was calculated on agarose gel pictures by GelAnalyzer 19.1 application. The pLDH weights were compared with a pLDH reference between 800–1000 base pairs by using an appropriate marker (27).

**Results**
Most of the goat blood samples contained no *h-Plasmodium* and only four samples were found positive (Table 1). The parasites were detected to be trophozoites of *P. vivax* and *P. falciparum* (Figure 1) (18). The *P. falciparum* samples...
determined positive by previous microscopic test were also found positive PfLDH by PCR method (Figure 2).

Discussion
The study site had long been known as a malaria-endemic area (28,29). The last reported local case occurred in October 2018 (30). Great efforts had been made by the government, including treatment of the confirmed malaria-positive patients and execution of a vector control program by performing an insecticide residual spraying (IRS) for the patient's house and surrounding areas (31). The distribution of long-lasting insecticide-treated nets (LLINs) has also been implemented (32). The main program of the Purworejo District Health Office to control malaria is the ‘Migration Surveillance’ (33). All programs were very successful in reducing malaria cases in Purworejo to zero until early 2021 (34). In June 2021, however, a significant increase in malaria cases was detected in Bener sub-district (35).

The efforts for controlling malaria have been comprehensive, targeting the patient and the vector of transmission. However, there is one more neglected factor, that is, the foraging behaviour of female Anopheles vectors in Purworejo. The previous vector survey examined three species including Anopheles maculatus, Anopheles subpictus, and Anopheles aconitus in Jatirejo village (36). These species are zoo-anthropophilic – in other words, they like animal blood as well as human blood (13,37,38). The bite prevention strategies to ensure human health included the execution of IRS program (31) and distribution of LLINs (32); however, no similar strategies were developed to protect animals against Anopheles bites. The zoophilic Anopheles gives malaria parasites opportunities to transfer from mosquitoes to animals.

The discovery of PfLDH in the blood of Etawa crossbreed goat is an important piece of information. The presence of the pLDH enzyme in the blood indicates a biological activity of the parasite's life that converts pyruvate into lactic acid. The pLDH enzyme can be produced by all five species of h-Plasmodium (39–41). Detection of pLDH has a sensitivity of up to 60.1% (42), and even a sensitivity of 94% has been reported (43). The specificity of pLDH has been found to be better than that of the HRP2 enzyme (44). The emerging of pLDH was easily detected in blood samples with parasitemia levels of 0.2-10% (45,46) and 75 parasites/µL (47,48).

Several assumptions made about the presence of pLDH in the blood of Etawa crossbreed goat have been found valid, suggesting that h-Plasmodium already inhibited its body. The pLDH enzyme is formed on the first day after erythrocytes is invaded by merozoites from the liver (7). Then the level of enzyme increases continuously until it reaches a peak at 24-48 hours in the erythrocytic phase (49,50) and, as a result, the enzyme becomes detectable in the blood. The process of pLDH formation in the human host continues until the beginning of gametocyte stage and its presence can still be detectable several days after treatment (9) or, put it another way, as long as there are living parasites in the blood. The results from studies on livestock groups have not been widely disclosed, but the above-mentioned findings may apply to human case.

The local case had not been detected in the study site in the previous three years, but it was recommended that everyone should remain vigilant. The detection of PfLDH in goat blood indicated that live parasites had already inhibited the animal before the present study was conducted. This implied that people of Jatirejo village were still under the threat of malaria disease. Therefore, it was
recommended that human contact with vectors at night should be strictly minimized. It was found that executing IRS programs in the focus areas as well as improving the efficacy of the distributed LLINs mosquito nets were likely the best solutions to deal with the problem. It was also suggested that the public awareness about the disease should be constantly promoted in order to help people adopt healthy behaviors and avoid the risks of malaria transmission.

**Conclusion**

In sum, the discovery of PfLDH in Etawa crossbreeds was a piece of interesting, valuable information that needed to be further analysed. The presence of PfLDH in the blood of Etawa Crossbreed goat was suggestive of the presence of infectious zoophilic vectors at the study site. Therefore, it was highly recommended that a continuing effort should be undertaken in order to monitor the biological activity of the human *Plasmodium* in domestic animals.

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**Authors’ Contribution**

DS: conception, data collecting, writing manuscript; SY: study design, methods, data analysis; YG: data interpretation, writing manuscript; MM: methods, writing manuscript; WH: laboratory analysis, data interpretation; and TDK: data collecting, data interpretation.

**Conflict of Interests**

The authors declare that they have no conflict of interests.

**Ethical Issues**

Ethical clearance number 029/EC/FK/2020 was issued by the Ethical Issues. The authors declare that they have no conflict of interests.

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