Human Plasmodium in Domestic Animals in West Sumba and Fakfak, Indonesia

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Research

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Abstract

Background: Although maximum efforts have been made, malaria in several areas in Indonesia is still high. This study aims to detect the possibility of *a Plasmodium* reservoir in domestic animals in endemic malaria areas.

Methods: Blood from the domestic animal was collected by EDTA tube, smeared and stained by Giemsa for detecting *Plasmodium* microscopically. Ten µl blood from EDTA tube dripped into filter paper for *Plasmodium* DNA capture. Nested PCR was used for the molecular detection of parasites, and DNA was sequenced from PCR products to ascertain *Plasmodium* species.

Result: A total of 208 and 62 animal blood samples were collected from Gaura and Fakfak villages. Thirty-two of 270 animals contained *P. falciparum* or *P. vivax*, and all are from Gaura village. The percentage of *Plasmodium* in buffalo, horse, goat, and dog is 20.7%, 14.3%, 5.8%, 16.7%, respectively. Neither *P. knowlesi* found in all samples, nor parasite detected in 18 pig blood samples.

Conclusion: Human *Plasmodium* exists in domestic animals in Indonesia. This finding may partly explain the persistence of the high prevalence of malaria in some endemic areas in Indonesia and may affect public health and malaria control strategy.

Background

Malaria is a disease caused by *Plasmodium* and transmitted by *Anopheles* mosquitoes. Four types of *Plasmodium* had been known to cause pathology in humans, namely *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. In recent years *P. knowlesi* has also been reported infects humans in Southeast Asia (1, 2, 3).

Before molecular technology developed, the known host for *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* are only humans. However, for the last two decades, several studies that aimed to analyse *Plasmodium*'s origin reported that those parasites originated from the animal. *Plasmodium falciparum* originated from gorilla (4) and chimpanzee (5, 6), *P. vivax* derived from African apes (7), *P. ovale* in humans and chimpanzees are genetically identic (8), *P. malariae* in chimpanzees (6) and *P. knowlesi* from monkeys (9, 10). Several factors have been hypothesized to explain this situation, including the loss of primates habitats and humans' aggressivity in exploring the forest (11). Epidemiology study has shown the contribution of forest workers to malaria incidence nearby villages (12).

East Nusa Tenggara and West Papua are known as malaria-endemic areas in Indonesia. In 2015 the annual parasites incidence (API) in West Papua and East Nusa Tenggara provinces was 31.29% and 7.04%, respectively (13). In 2018 the API in Fakfak West Papua was 4.85%, while in West Sumba, East Nusa Tenggara was 12.9% (14). Due to the high rate of API in these areas, we are interested in exploring the presence of human *Plasmodium* in the domestic animal in those two malaria-endemic areas.

Material And Methods

Study area and population

This research was conducted in Gaura village, West Sumba Regency in 2018, and Fakfak, West Papua Province, in 2019 (Fig. 1). Gaura's area is 29.96 km2 wide and inhabited by 9,584 people (15). Fakfak has 11,036 km2 in size and inhabited by 84,692 people (16). The main occupation of residents in both locations was farmers, and almost every family has livestock such as goats, horses, cows, pigs, and buffalos, whose cages are located around the residential. The residents also have pets such as dogs and cats that freely in and out of the house.

Sampling collection

Sampling collection was done with the help of a veterinarian and staff from the Animal Husbandry Department. The blood was drawn using a 5 ml EDTA tube. A vacutainer needle 16–18 in size was used to collect blood from buffaloes, goats, pigs, and horses from the jugular vein located in the neck's ventrolateral part. The dog's blood was drawn using a 21 size vacutainer needle and bleed from *the cephalic antebrachial* located in the leg. For the *Plasmodium* microscopic identification, approximately 10 ul of EDTA blood was dropped onto a glass object, smeared and stained with Giemsa. The remaining blood was dripped onto filter paper (Whatman CAT No. 1442-090) until the blood was absorbed with approximately 1.5 cm in diameter. The dry filter paper was put in a sterile plastic clip and stored at room temperature for a maximum of 10 days.

DNA extraction

A dried blood spot (DBS) isolation kit (Cat. 36000) from Norgen Biotec was used for filter paper DNA extraction. A 6 × 3 mm filter paper containing blood was put into a 1.5 ml tube containing 100 µl of digestion buffer B. The tube was then vortexed and incubated at 85 °C. Twenty µl of proteinase K and 300 µl of lysis buffer B was added to the tube and then vortexed before incubated at 56 °C. All incubation processes were carried out for 10 minutes. Two hundred and fifty µl of 95% ethanol was added to the tube and then vortexed. The tube's DNA was washed by adding 500 µl of WN wash solution and then centrifuged for 1 minute at a speed of 8000 rpm. The second washing was carried out twice with 500 µl washed with wash solution and centrifuged at 14000 *rpm*. For DNA elution, 90 µl of elution buffer B was put into the tube and centrifuged at 8000 rpm for 1 minute. The purified DNA was stored at -20ºC.

DNA Amplification and electrophoresis

DNA amplification and electrophoresis were performed by following procedures as directed by Tiangen. For details, *Plasmodium* DNA amplification was carried out used the Nested PCR method with a 2x Tag Plus PCR mix (Tiangen) enzyme. The final volume of each sample was 12.5 µl, which contained 6.25 µl enzyme, 2.25 µl ddH2O, 1 µl forward-, 1 µl reverse- primers, and 2 µl DNA samples. For sequencing, the volume of mixtures was double, with the final volume of 25 ul. In table 1, the primer sequences can be seen.

For Nested 1 DNA amplification, the temperature was set up as follows: Denaturation 94ºC (1 minute), annealing 55ºC (1 minute), extension 72ºC (1 minute) in 35 cycles. For Nested 2, denaturation was carried out at 94ºC (30 seconds) and extension at 72ºC (30 seconds) in 35 cycles. There was a difference in annealing temperature for each species in Nested 2; 55 °C (1 minute) for PCR multiplex *P. falciparum* and *P. vivax*, while for *P. knowlesi* 56 °C (1 minute). Nested one products were used as templates for Nested two. Nested one and Nested two products were run by 1.5% and 2%, respectively, for electrophoresis. We did not perform molecular work for *P. ovale* and *P. malariae* since we have difficulty finding the positive control, and according to the local health office, these species have never been reported from Sumba and Fakfak.

Sequencing and alignment

To determine *Plasmodium*'s species, Nested two products that had positive bands were sent to Genetics Science in Singapore to be requested. Results of DNA sequences adjusted using multiple alignments found in the Bioedit application (17). The results were then read by the BLAST program from the NCBI website (18).

Results

A total of 208 and 62 animal blood samples were collected from Gaura and Fakfak villages, respectively. The samples consisted of 92 buffalos, 21 horses, 121 goats, 18 dogs, and six pigs. By using the microscopic and Nested PCR methods, we found 32 of 270 animals were carrying *P. falciparum* and or *P. vivax*. The percentage of animal that positive for *Plasmodium* as follow: Buffalo 20.7%, horse 14.3%, goat 5.8%, dog 16.7%. One buffalo have mixed infection (*P. falciparum* and *P. vivax*). There was no any *P. knowlesi* found in all samples and no *Plasmodium* in 18 pig blood samples. The display of PCR gel products, the sequences of DNA results, and the sample's quality can be seen in Figs. 2, 4, and 5, respectively. The forms of trophozoites, schizonts, and gametocytes microscopically at 100 times magnification can be seen in Fig. 3. Apart from dogs, the erythrocytes in animal samples appeared to be smaller than the human erythrocytes. All blood that was containing *Plasmodium* were collected from Gaura. The distribution of *Plasmodium* species in animals' blood samples from Gaura and Fakfak can be seen in table 2.

Discussion

The suspicion of *Plasmodium* presence in domestic animals arose because malaria cases in those two villages remained high even though many efforts, including insecticide-treated bed nets, have been applied. This study identified 32 of 270 animal blood (11.9%) contained *Plasmodium*, known as human parasites.

The previous studies found *Plasmodium relictum* in avian (19), *P. cephalophi* in ungulate (20), *P. traguli* in mousedeer (21), *P. brucei* in gray duiker (22, 23), *P. bubalis* in water buffalo (24), and *P. odocoilei* in white-tailed deer (25, 26). In ruminant, *P. caprae* was found in goat (27), in Rodentia was *P. bergei* (28), and in

primates were found *P. cynomolgi*, *P. inui*, and *P. fragile* (29). The five *Plasmodium* that infect humans were originally parasites in primates (1, 2, 6, 7, 8, 9, 30). In this study, we found *P. falciparum* in buffalo, goats, dogs, *P. vivax* in buffalo, goats, dogs, and horses. At first, we were not so sure about the presence of *Plasmodium* in these animal erythrocytes. However, the Nested PCR showed the same results for all positive samples. The sequencing results of the positive bands in Nested 2 showed that the bands were *P. falciparum* and *P. vivax* (Fig. 7). This is the first investigation reporting human *Plasmodium* in domestic animals (ruminant, ungulate, and carnivore).

We have considered the possibility of contamination. Therefore, we re-extracted DNA from the same filter paper's blood, and the results were no different from the first Nested PCR. We also performed PCR using other primers, namely rPF1, rPF2, and primers rPV1, rPV2 (31) to detect *P. falciparum* and *P. vivax*, and the results were still positive.

The discovery of *Plasmodium* in buffaloes, goats, horses, and dogs in malaria-endemic areas raises the following questions. How did P. falciparum and P. vivax live in these pets? Are they intermediate hosts for this parasite? Did these *Plasmodium* species evolve to live in ruminants, ungulates, and carnivores? As a result of repeated exposure, have these animals become more permissive to *Plasmodium*, which generally lives in humans? Is this parasite pathogenic in animals? As is known, *P. knowlesi* is a commensal microbe in primates but is pathogenic in humans (1, 2, 3). In *P. knowlesi*, migration from primates to humans can be caused by loss of forests or human invasion of primate habitat (11). It might be possible that the proximity between animals and humans makes it easier for mosquitoes to transfer human parasites to animals and vice versa.

Although both Fakfak and West Sumba have high API, only animals in West Sumba (Gaura village) have human *Plasmodium*. This difference may be due to the distance between the human house and animal cages. In Fakfak, the cages are separated approximately 50–500 meters from the main house, while in Gaura, people generally live in a stilt house where the ground floor functions as animal sheds. This condition allows microbial to be transferred by mosquitoes between humans and animals. Unlike in Gaura, the sampling locations in Fakfak are far from each other, and the steep geographical conditions in Fakfak made it difficult for us to collect as many samples as we got from Gaura. This may indirectly lead to the absence of *Plasmodium* found in Fakfak domestic animals.

Although the use of a microscope can detect *Plasmodium*'s presence well, due to the size of erythrocytes, which are generally smaller in animals than humans, the molecular method becomes significant in detecting the presence of *Plasmodium*. In this study, Nested PCR was used to detect *Plasmodium* because its sensitivity was as high as Real-Time PCR and its relatively lower cost (32, 33). For further studies, the recommended microscopic method of using a double fluorescent dye with Giemsa's stain may need to be considered (34).

Conclusion

This study found *Plasmodium* species in domestic-animals (ruminants, ungulates, and carnivores) previously known as human parasites. These results may partly answer the question of why malaria is challenging to eliminate in an area. It is hoped that these findings can be applied to improve public health and become a reference in formulating malaria prevention strategies.

Declarations

Acknowledgments

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Ethical clearance

This study was approved for ethical clearance by the ethics committee of the Faculty of Medicine, Hasanuddin University (734/H4.8.4.5.31/PP36-KOMETIK/2018)

Competing Interests

The authors declare that we have no competing interest of financial, professional or personal interests that might affect the performance or presentation of the work described in this manuscript.

Authors' contributions

MM conceived, designed the experiments, performed the experiments, analysed the data, and wrote the paper. SW conceived, designed the experiments, analysed the data, and wrote the paper. IW contributed in molecular analysis and wrote the paper. FH contributed in molecular analysis. All authors read and approved the final draft of manuscript.

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Tables

Due to technical limitations, table 1-2 is only available as a download in the Supplemental Files section.

Figures

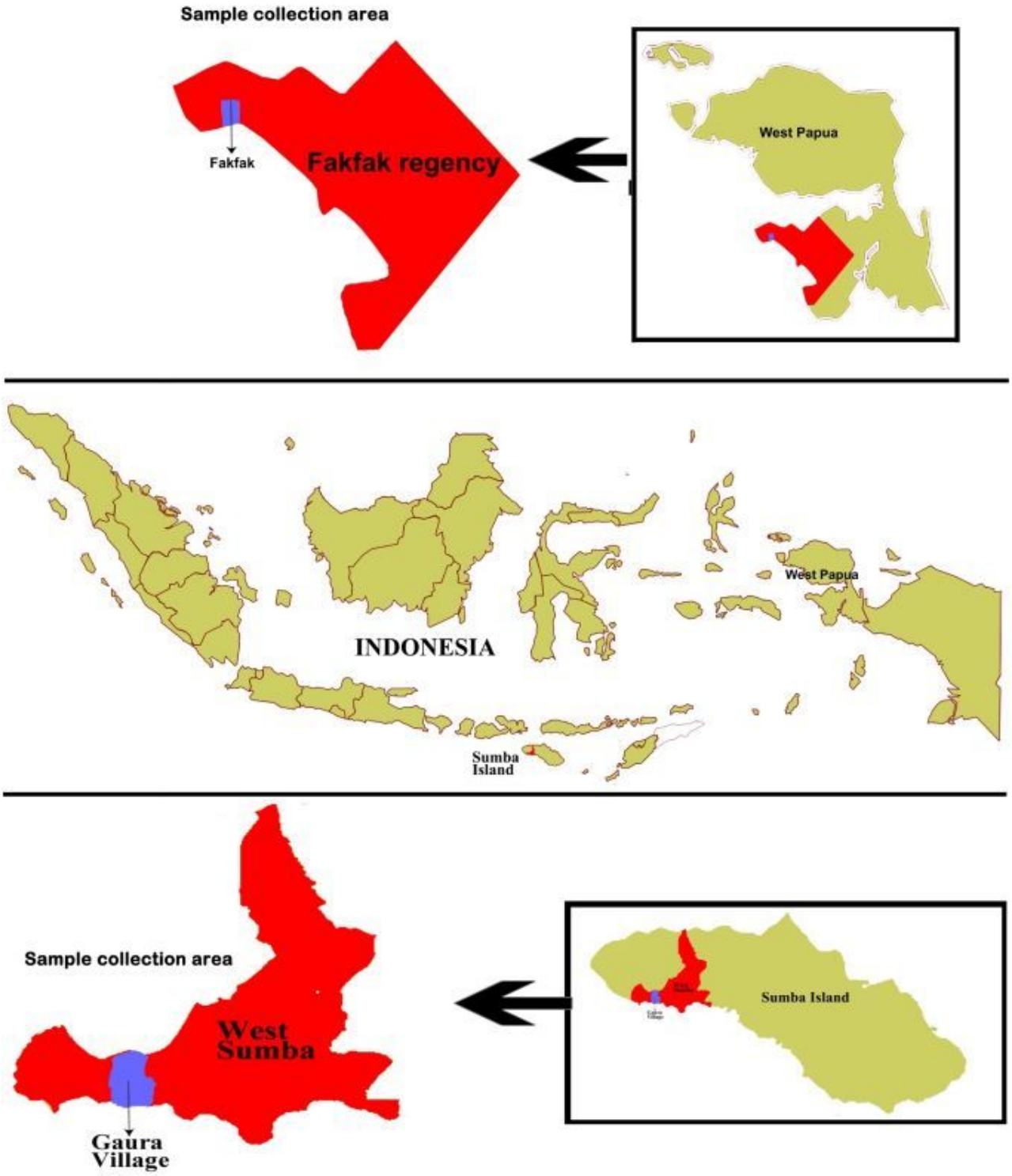

Figure 1

Study area; Map of Gaura Village, West Lamboya, West Sumba Indonesia and Fakfak regency, West Papua Indonesia.

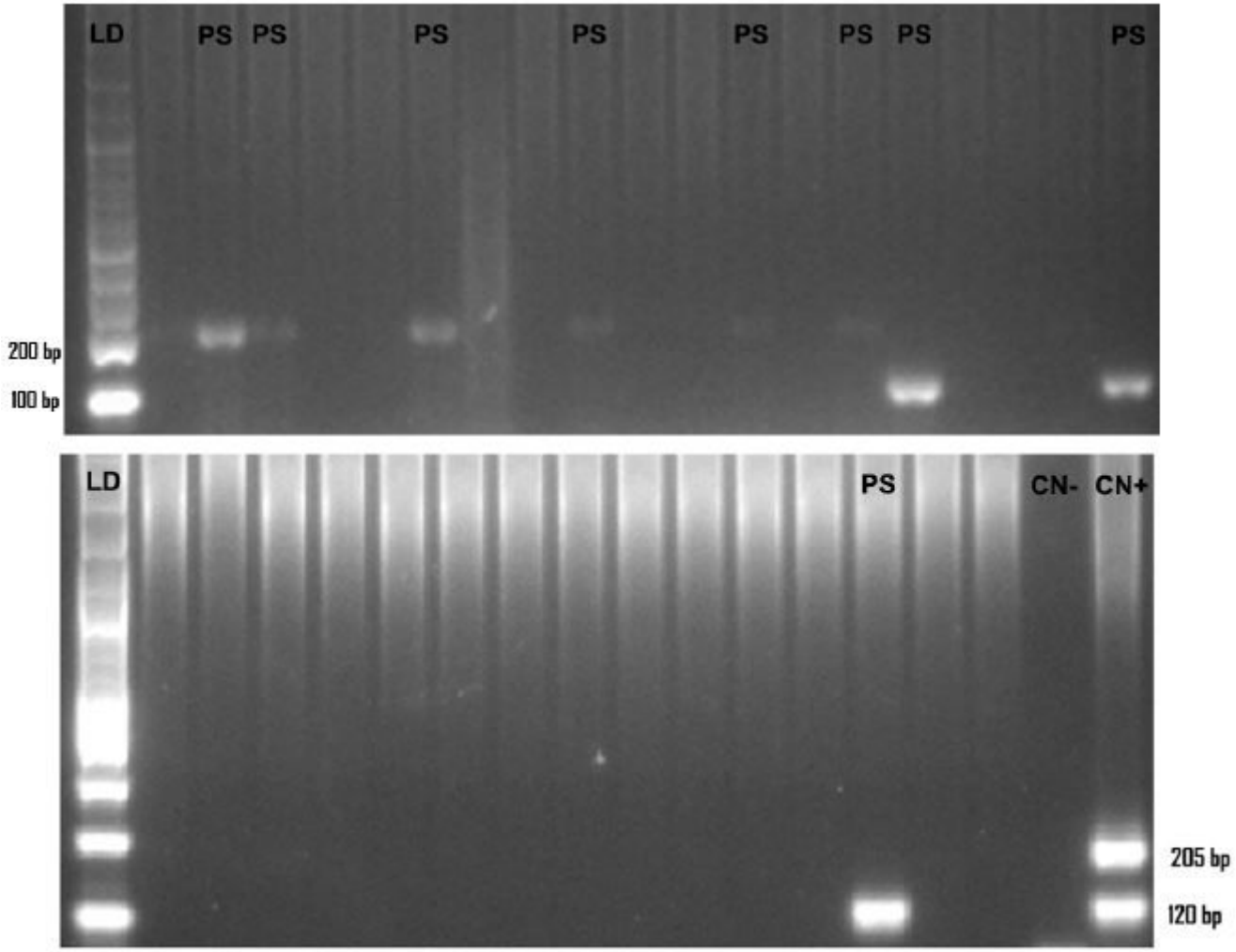

Figure 2

Gel view of PCR product from Plasmodium vivax and Plasmodium falciparum in domestic animals in Gaura, West Sumba (LD=DNA ladder, PS=positive samples, CN=control negative, CP=control positive) by Nested PCR (multiplex PCR). 120 bp for positive Plasmodium vivax, 205 bp for positive Plasmodium falciparum.

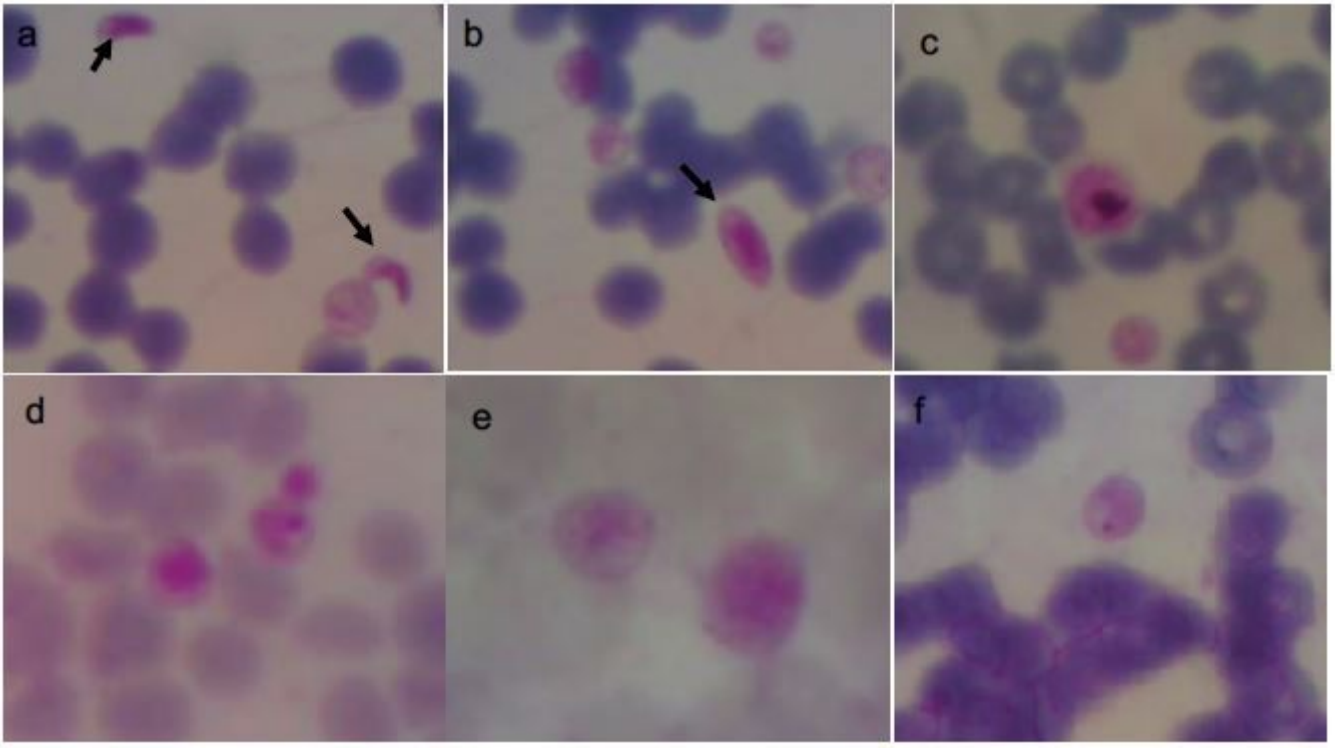

Figure 3

Morphology of Plasmodium in animal from Gaura village. Gametocytes (a,b) and schizon (c) in buffalo, schizont in goat (d), schizont in dog (e) and trophozoite in horse (f) with Magnification 100 times.

Sample buffalo (*Plasmodium falciparum*)
TAAGGTTTTCTAATAAATTATGTTTTTATCAGATATGACAGAATCTTTTTTAAAATCTCTTCAATATGCTT
TTATTGCTTTTGAGAGGTTTTGTTACTTTGAGTAAAATTAAGTGTTCATAACAGACGGGTAGTCATGAT
TGAGTTCATTGTGTA

Sample goat (*Plasmodium falciparum*)
TAAGGTTTTCTAACAAATTATGTTTTTATCAGATATGACAGAATCTTTTTTAAAATCTCTTCAATATGCT
TTTATTGCTTTTGAGAGGTTTTGTTACTTTGAGTAAAATTAAGTGTTCATAACAGACGGGTAGTCATGA
TTGAGTTCATTGTGTA

Sample dog (*Plasmodium falciparum*)
TAAGGTTTTCTAATAAATTATGTTTTTATCAGATATGACAGAATCTTTTTTAAAATCTCTTCAATATGCTT
TTATTGCTTTTGAGAGGTTTTGTTACTTTGAGTAAAATTAAGTGTTCATAACAGACGGGTAGTCATGAT
TGAGTTCATTGTGTAT

Sample horse (*Plasmodium falciparum*)
AATAACTTATGTTTTTATCATATATGACAGAATCTTTTTTAAAATCTCTTCAATATGCTTTTATTGCTTTT
GAGAGGTTTTGTTACTTTGAGTAAAATTAAGTGTTCATAACAGACGGGTAGTCATGATTGAGTTCATT
GTGTAAGA

Sample horse (*Plasmodium vivax*)
TGCGCATTTTGCTATTATGTGTTCTTTTAATTAAAATGATTCTTTTTAAGGACTTTCTTTGCTTCGGCTT
GGAAGTA

Figure 4

Plasmodium DNA sequence alignments from Gaura village by ClustalW multiple sequence alignment.

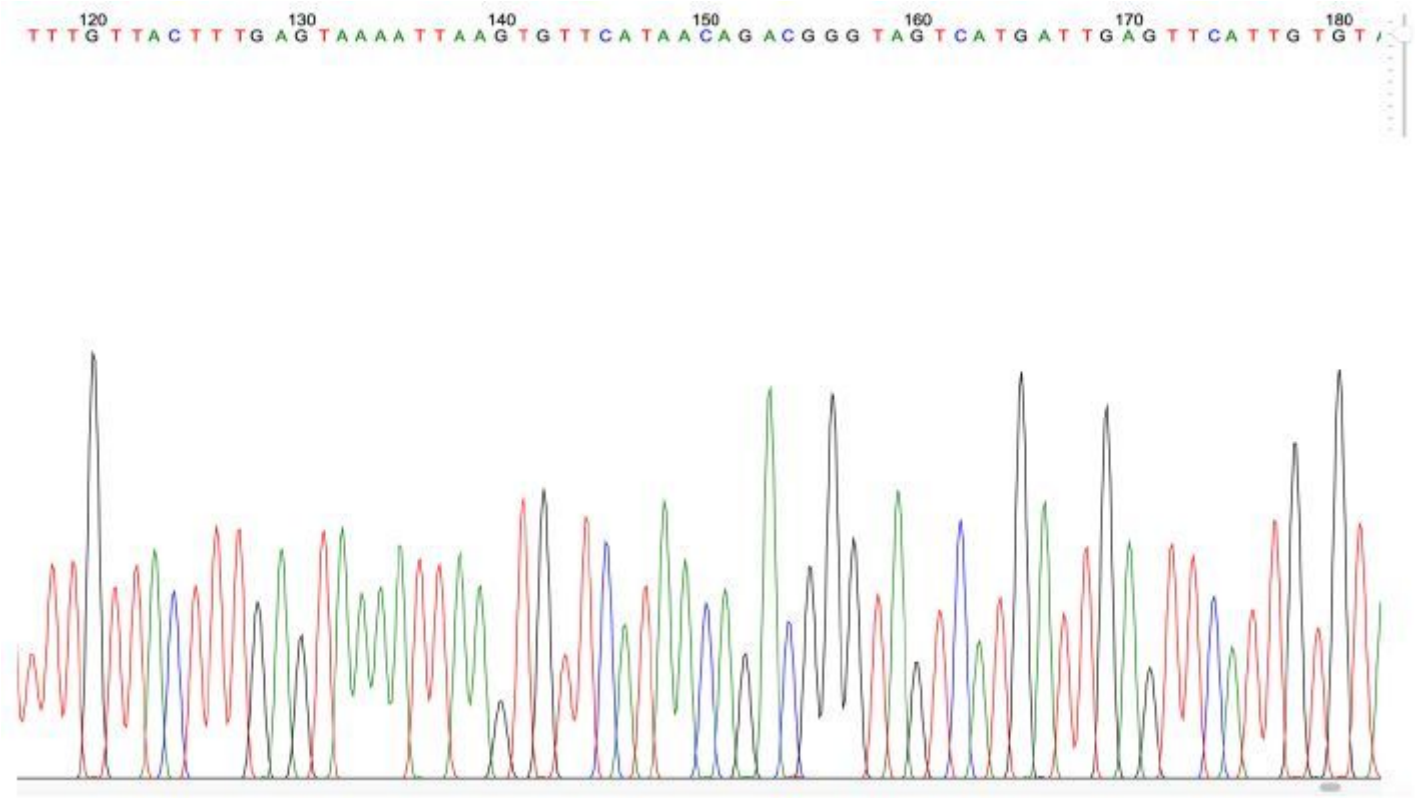

Figure 5

Example of Plasmodium PCR product quality from Gaura village.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1and2.xlsx