Molecular detection of Anaplasma phagocytophilum DNA in Olive Baboons and Vervet monkeys in Kenya

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Research Article

Keywords: Zoonosis, Olive baboons, Vervet monkeys, Kenya, Anaplasma phagocytophilum

Posted Date: April 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-355087/v1

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Molecular detection of *Anaplasma phagocytophilum* DNA in Olive Baboons and Vervet monkeys in Kenya.

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Abstract

Background

Nonhuman primates (NHPs) occupy an important place in zoonotic spill-overs, serving as either reservoirs or amplifiers of multiple neglected tropical diseases including tick-borne infections. Anaplasmosis is caused by obligate intracellular bacteria of the family *Anaplasmataceae*. They are transmitted by Ixodid tick species and have a wide host range including wild animals, domestic animals and humans. The aim of this study was to establish the presence of *Anaplasma phagocytophilum* in Olive baboons and Vervet monkeys in Laikipia County, Kenya.

Results

A total of 164 whole blood samples, collected by USAID Predict II project from Laikipia County, Kenya, were included in the study. These comprised of 146 samples from Olive baboons (*Papio anubis*) and 18 from Vervet monkeys (*Chlorocebus pygerythrus*) respectively from Mpala Research Center and Ol jogi Conservancy in Laikipia County. Using conventional Polymerase Chain Reaction (PCR), positive results for *A. phagocytophilum* in 26 Olive baboons and 4 Vervet monkeys were detected with primer sets EHR16SD/R. However, low sensitivity was observed with the p44 gene. The amplification of DNA template with the primer set p44 (p3709 5/ p4257 5) using nested PCR could not be obtained. Our results revealed the presence of *A.
phagocytophilum in Olive baboons and Vervet monkeys. This study found an overall prevalence of 18.3% for Anaplasma phagocytophilum. A distinct genotype of A. phagocytophilum was detected that was different from the others in the gene bank database.

Conclusion
This study provides valuable information on the presence of A. phagocytophilum bacteria in Olive baboons and Vervet monkeys in Kenya. It indicates a need for future research to establish the public health implications of zoonotic A. phagocytophilum isolates and the role of nonhuman primates as reservoirs.

Key words: Zoonosis, Olive baboons, Vervet monkeys, Kenya, Anaplasma phagocytophilum
Background

There has been a rise in the frequency of emerging infectious diseases (EIDs), among which zoonotic tick-borne infections especially rickettsial diseases such as anaplasmosis, are implicated [1]. What most of the recent pandemics have proven is that emerging infectious diseases are mainly of animal origin, particularly wildlife [2]. The complex interaction among wildlife, livestock and human populations is a factor that contributes to their emergence [3].

Among wildlife species, non-human primates are often proprietors to different microbial agents some which have zoonotic potential. Primates are closely related to humans phylogenetically and ecologically [4] and they can indirectly transmit infectious agents to humans through intermediate hosts, arthropod vectors or directly through bush meat consumption and accidental bites [5]. Certain factors such as forested tropical regions experiencing land-use changes and encroachment as well as those with a high wildlife biodiversity facilitate the spread of these diseases to livestock and man [6]. Others include adoption of new technology in farms, destruction of habitats, climate change, travel and encroachment into new habitats [2].

This study focused on *Anaplasma phagocytophilum*, a pathogenic bacterium of zoonotic potential often spread among wildlife then to livestock and man [7]. *Anaplasma* hemoparasites belong to the family of *Anaplasmataceae*, order of Rickettsiales, class Alphaproteobacteria and genus *Anaplasma* [8]. However, reclassification of *Anaplasma* happened recently resulting in *A. phagocytophilum* being included in the genus whose members were only pathogens with host specificity to ruminants such as *A. marginale*. This followed advancement in genetic analyses of 16S rRNA genes, groESL and surface protein genes. There is a considerable strain variation with *A. phagocytophilum* such that there appears to be existence of serological cross-reactivity [9], a
minor degree of variation in the nucleotide sequences of the 16S rRNA, groESL, gltA, ank, and msp2 genes [10] and a difference in the host infectivity [11].

This bacterium has a wide host range including domestic animals, wildlife and man [12]. In man the disease is known as human granulocytic anaplasmosis (HGA) [13]. The disease often presents with influenza-like symptoms in animal and human hosts which include fever, anorexia, diarrhea, leukopenia and thrombocytopenia [7]. Ixodid ticks are important in their maintenance as vectors [14]. The emergence of Anaplasmataceae as human pathogens has gained the attention of scientific community. A recent surveys have shown human infection with anaplasmosis including in Venezuela [15] and one in Morocco where dog owners were infected with *A. phagocytophilum* [16].

Recent reports on animal anaplasmosis are available from France, Massachusetts, Brazil, Zambia Ethiopia and Kenya in both domestic and wild animals [17-22]. While *Anaplasma* in NHPs has been reported in some countries, its importance in NHPs in Kenya is not yet known which has therefore led us to investigate the occurrence of this bacteria in NHPs in Laikipia, Kenya.

The investigation focused on blood DNA presence of *A. phagocytophilum* in Olive baboons (*Papio anubis*) and Vervet monkeys (*Chlorocebus pygerythrus*) in Laikipia County, Kenya. Laikipia is part of Kenya’s rangelands mainly inhabited by trans-human pastoralists. It also has a large wildlife population including primate species. These animals are found close to human settlements.

The objectives of this present study were (i) to assess the prevalence of *A. phagocytophilum* in Olive baboons and Vervet monkeys and (ii) to establish whether the bacteria is genetically diverse.

**Methods**

*Study area, sample population and sample size*
This study was part of the USAID Predict II project whose aim was to collect targeted information to support the interventions to mitigate spread of zoonotic viruses with pandemic potential. The focus was on highest risk locations and interfaces, where animals and people share changing landscapes. The study area, Laikipia County (Figure 1) located in the Rift Valley of Kenya with co-ordinates of 005°N 36040’E, was picked as one of the locations. There is a diverse range of wildlife in the area, including NHPs with 8 species of them found in the area.

Figure 1: Map of the sampling sites.

This study focused on two NHP species: Olive baboons (*Papio anubis*) and Vervet monkeys (*Chlorocebus pygerythrus*), since they are closely related to humans phylogenetically and are implicated in most of the listed emerging human pathogens. The sampling sites Mpala research Centre and Ol jogi Conservancy (Figure 1) were conveniently chosen as large numbers of these species inhabit these sites. All the whole blood samples from wild Olive baboons and Vervet monkeys that had been collected by USAID Predict II project from Laikipia County were included in this study constituting the study’s sample size of 146 Olive baboon and 18 Vervet monkey. The animals were not owned.
**DNA extraction**

Whole blood samples stored in TRIzol reagent at -80°C were retrieved and allowed to thaw at room temperature. Extraction of genomic DNA from each of the whole blood sample was done using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA) following manufacturer’s instructions.

**Nested PCR**

First set of primers (p3709-p4257) were used in a nested PCR run for amplifying the p44 gene of *Anaplasma* species in Olive baboons and Vervet monkeys (Table 1). Using the second set of primers (p3761-p4183), 1ul of the product from the first amplification was used in a 25ul reaction mixture. Reactions were performed in a final volume containing 18.25ul double distilled RNase/DNase free water, 2.5ul PCR buffer, 0.75ul 1.5Mm Mgcl\(_2\), 0.5ul of 10um DNTPs, 0.5ul of 10um forward primer, 0.5ul of 10um reverse primer, 0.1ul of 2.5U Taq DNA polymerase and 1.0ul of the DNA template. Thermal cycling profiles were as previously described [23].

**Conventional PCR**

The primer set EHR16SD/R was used in a simple PCR to amplify 345bp fragment of *Anaplasma* 16S rRNA gene on the same samples with thermo-cycling profile as previously described [24]. PCR products were electrophoresed on 1% agarose gel to check the size of amplified fragments by comparison with a DNA molecular weight marker (1 Kb Plus DNA Ladder, Promega, Madison, USA).

**DNA sequencing and data analysis**

Ten selected positive *Anaplasma* spp. PCR products obtained with primers EHR16SD/R were purified with Thermo Scientific GeneJET PCR Purification Kit#K0701, #K0702 Protocol
according to the manufacturer’s instructions. Purified DNA fragments were sequenced using an
ABI PRISM 377 Genetic Analyzer (Applied Biosystems, USA), using the same forward and
reverse primers (Table 1) of each PCR assay. Sequence assembly for forward and reverse primers
was done using DNA Sequence Assembler v4 (2013), Heracle BioSoft [25]. The sequences were
matched to those deposited in the GenBank database using BLAST search
(http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment of the sequences was done using
BioEdit Sequence Alignment Editor (Hall, T.A. 1999). Construction of phylogenetic tree was
done using Muscle 3.8 using the neighbor-joining method and visualization of the trees with
FigTree v1.4.4 [26].

All methods were carried out in accordance with relevant guidelines and regulations.

**Results**

**Molecular survey of Anaplasma species**

A total of 164 blood samples 146 from Olive Baboons and 18, Vervet monkeys where screened
for *A. phagocytophilum* through nested PCR employing the two sets of primers for p44 genes
(Table 1). However, all these samples tested negative. A re-run and troubleshooting was done to
confirm the results still turned out negative. With the same samples, a different assay and
conventional PCR using primers targeting 16S rRNA gene (Table 1), it amplified a specific band
of approximately 345bp. The overall prevalence for anaplasmosis was 18.3% (30/164) as
estimated by EHR16SD/R PCR (Table 1) with 17.8% and 22.2% in Olive baboons and Vervet
monkeys respectively.

**Table 1**: Primers used for detection and/or characterization of *Anaplasma* species in the present study
Molecular characterization of Anaplasma phagocytophilum. 16S rRNA genotypes

Nine of the 10 PCR products were successfully sequenced on both DNA strands and generated nucleotide sequences with primers EHR16SD/R targeting 345bp of the 16S rRNA gene of Anaplasma spp. Multiple alignment of Anaplasma nucleotide sequences of 4 A. phagocytophilum isolates revealed that all the sequences were conserved except one 15A which was the most divergent in comparison to the rest with a lot of accumulated mutations and numerous insertions. They all shared 99% to 100% nucleotide similarity except 15A at 81% (Table 2).

Table 2: BLASTn analysis results using 16S rRNA sequences of isolates from Olive baboons and Vervet monkeys.

<table>
<thead>
<tr>
<th>Samples sequences</th>
<th>Animal species</th>
<th>Homologous sequences</th>
<th>E values</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15A</td>
<td>Olive Baboons</td>
<td>A.phagocytophilum</td>
<td>5</td>
<td>81.65</td>
</tr>
<tr>
<td>41A</td>
<td>Olive Baboons</td>
<td>A.phagocytophilum</td>
<td>4</td>
<td>99.39</td>
</tr>
<tr>
<td>39A</td>
<td>Olive Baboons</td>
<td>A.phagocytophilum</td>
<td>3</td>
<td>99.39</td>
</tr>
<tr>
<td>28A</td>
<td>Olive Baboons</td>
<td>A.phagocytophilum</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>
The sequences of the *A. phagocytophilum* isolates from Kenya were identical to those from Japan, South Korea, France, China, South Africa and Denmark. The species isolates were from human, cattle, ticks, dogs and rodents. The accession numbers were JQ622148.1, MF351963.1, KU559922.1, MG519284.1, KX810088.1, MH122888.1, MK814411.1, MK814407.1, MF582329.1, MK814412.1, MK271308.1, MH122891.1, AY776165.1, KT986058.1, KU513793.1, and KR611718.1 (Figure 2). Phylogenetic analysis revealed that the isolates from Japan, South Korea, France, China, South Africa and Denmark belonged to clade I but have recent common ancestor with the Kenyan isolates clustered into one clade II except isolate 15A which appeared separately and represented an outgroup (Figure 3).

**Figure 2:** Multiple sequence alignment of 16S rRNA gene for *A. phagocytophilum* isolates.
Discussion

An epidemiological surveillance of pathogens in NHPs found at the human-animal interface is important in developing strategies on prevention and control of emerging and re-emerging zoonotic diseases.

Therefore, this study reports the detection of *A. phagocytophilum* DNA within the studied localities with an overall prevalence of 18.3%. *A. phagocytophilum*, an obligate intracellular bacterium, is the agent of human granulocytic anaplasmosis, formerly known as human granulocytic ehrlichiosis [13]. *A. phagocytophilum* has been previously reported to infect a wide range of animal hosts in various parts of the world [17-19], in Africa, [20, 27] while in Kenya [21, 22, 28, 29]. This study adds further evidence on the occurrence of *A. phagocytophilum* in Olive baboons and Vervet monkeys in Kenya.
In this study, a total of 164 samples were tested and an overall prevalence for anaplasmosis was 18.3% and 17.8% and 22.2% in Olive baboons and Vervet monkeys respectively which was similar to that reported in a survey on cattle in Ethiopia [27]. This is however inconsistent with reports by other authors. For example, in Zambia reported a 13% prevalence in baboons and Rhesus macaques [20]. In Kenya, a study on Anaplasma hemoparasites in wild animals revealed high seroprevalence [29] while another reported 35.5% sero-prevalence in cattle in Nairobi, Kenya [22]. Studies have shown that A. phagocytophilum can persist for the lifetime of animals due to its effect on the glucose metabolic pathways for maintenance of infection and multiplication [30]. This explains the high detection rates of Anaplasma infection in certain animal populations. Different host species, sample sizes, diagnostic technique, study areas demography and endemicity disease status in each study region could also explain it.

In this study, primers targeting p44 and 16S rRNA genes of A. phagocytophilum were identified from previous studies and used for detection of A. phagocytophilum [23, 24]. However, there was a significant difference in the performance of these two assays. The primers targeting p44 gene did not yield any positive bands on PCR. This is in agreement with several studies in cattle [27], wild birds and bats [31] and in ruminants and ticks [32]. This can be explained by the high variability of p44 gene, which includes intra-species variability, with consequent protein polymorphism and the generation of antigenic variations [33, 34]. The 16S rRNA gene yielded positive bands and therefore reinforced that it is a good marker for detection of Anaplasma as reported by other studies [20, 27, 35].

Further sequencing analysis revealed that the sequences of 16S rRNA were very conserved not only between African isolates but also between the other isolates of world-wide origin agreeably with previous studies [36]. The sequences of the A. phagocytophilum isolates from Kenya were
identical to those from Japan, South Korea, France, China, South Africa and Denmark. The species isolates were from human, cattle, ticks, dogs and rodents (Figure 3). This is consistent with a study on baboons in Zambia [20]. Multiple alignment of *Anaplasma* nucleotide sequences of the *A.phagocytophilum* isolates revealed that all the sequences of samples from baboons were conserved except for 15A. It was the most divergent in comparison to the rest with a lot of accumulated mutations and numerous insertions (Figure 2).

Extensive molecular studies have enabled understanding of the genetic profiles, level of genetic relatedness or variations among isolates across the different geographical regions and animal species. The isolates from Japan, South Korea, France, China, South Africa and Denmark belonged to clade I but have recent common ancestor with the Kenyan isolates clustered into one clade II except isolate 15A which appeared separately and represented a kind of outgroup (Figure 3). Previous studies have reported genetic relatedness between *A. phagocytophilum* infecting animals and humans [15, 35]. Similarly, in this study isolates from Olive baboons appear to be genetically related to those of human (MF582329.1) (Figure 3).

This, to the best of our knowledge, this is among the first molecular detection and estimate of the prevalence of *Anaplasma phagocytophilum* in Kenya’s Olive baboons and Vervet monkeys. Non-human primates have been shown to host different pathogens, including several *Anaplasma* species [34, 20]. We assume that they could serve as a good indicator of bacteria circulation in ecosystem and explain the persistence of anaplasmosis in domestic animals despite consistence control.

**Conclusions**

This paper reports the presence of novel *Anaplasma phagocytophilum* in Olive baboons and Vervet monkeys in Laikipia County, Kenya. These findings open new concerns about the specificity of the molecular assays used to detect different *Anaplasma* species in and provide useful molecular
information to elucidate the evolutionary history of bacterial species related to *A. phagocytophilum*. Further studies are needed to investigate the role of Olive baboons and Vervet monkeys in the maintenance of *Anaplasma* organisms to establish the public health implications of zoonotic *A. phagocytophilum* isolates.

**Abbreviations**

- **NHPs**: Nonhuman primates
- **USAID**: United States AID
- **PCR**: Polymerase chain reaction
- **DNA**: Deoxyribonucleic acid
- **RNA**: Ribonucleic acid
- **rRNA**: Ribosomal Ribonucleic acid
- **EIDs**: Emerging infectious diseases
- **HGA**: Human granulocytic anaplasmosis
- **OR**: Odds ratio
- **CL**: Confidence level
- **CI**: Confidence interval
- **DNTPs**: Deoxyribonucleotide triphosphate
- **BLAST**: Basic Local Alignment Search Tool
- **Msp**: Major Surface Protein


descriptions of six new species combination and designation of Ehrlichia equi and ‘HGE agent’


**Declarations**

**Ethical approval**

Approval for this study was given by the Institute of Primate research Institutional Scientific and Ethics Review Committee (ISERC): Ethical approval number ISERC/T01/18, 17 November. Since the information collected for this study was not of a sensitive nature and non-invasive as only blood samples previously collected were used, the ethical approval and guidelines received from this committee was deemed sufficient to conduct the study.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Data and materials are available upon reasonable request from the corresponding author.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

The work was supported by the funding from the USAID Predict II project, 2018.

**Authors’ Contributions**
SJM conducted the field and laboratory work. In addition, she participated in the study design, did the data analysis and preparation of this manuscript. GMM, JMK, and TAO were involved in the conception of the study, study design and the writing of this manuscript. SKM participated in the laboratory work. All authors have read and approved the final manuscript.

Acknowledgement

We thank the staff at the Molecular Laboratory at the Institute of Primate Research, Kenya who assisted with laboratory analysis of the work. The authors would like to thank Dr. Mercy Akinyi, and Dr. Lilian Waiboci for their invaluable contribution in the work.

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Map of the sampling sites. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.
Figure 2

Multiple sequence alignment of 16S rRNA gene for A. phagocytophilum isolates. The conserved regions are represented by the dots (.) while the variable regions are indicated by the letters representing the nucleotide A, C, G and T.

Figure 3

Phylogenetic tree of the 16S rRNA gene from A. phagocytophilum isolates.