Anaplasma marginale and A. platys Characterized from Dairy and Indigenous Cattle and Dogs in Northern Vietnam

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Abstract: Anaplasma marginale and A. platys were detected and characterized (16S rDNA sequence analysis) from dairy and indigenous cattle, and the latter in domestic dogs in Vietnam. A phylogenetic tree was inferred from 26 representative strains/species of Anaplasma spp. including 10 new sequences from Vietnam. Seven of our Vietnamese sequences fell into the clade of A. marginale and 3 into A. platys, with strong nodal support of 99 and 90%, respectively. Low genetic distances (0.2-0.4%) within each species supported the identification. Anaplasma platys is able to infect humans. Our discovery of this species in cattle and domestic dogs raises considerable concern about zoonotic transmission in Vietnam. Further systematic investigations are needed to gain data for Anaplasma spp. and members of Anaplasmataceae in animal hosts, vectors and humans across Vietnam.

Key words: Anaplasma marginale, Anaplasma platys, hybrid dairy cattle, indigenous cattle, domestic dog, phylogeny, 16S rDNA, Vietnam
Single 16S rDNA sequences have been successfully explored for taxonomic clarification of *Anaplasma* spp. and *Ehrlichia* spp. in many endemic regions/countries [2,6,8,12,13,17].

Over 2 years (May 2015 to August 2017), 226 blood samples were collected from cattle and domestic dogs in 2 provinces of the Red River Basin in the north of Vietnam. In the upland Bavi district in Ha Noi (21°5’0”N/105°23’0”E), 78 samples were obtained from hybrid dairy cattle used for milk production, 66 from indigenous cattle and 14 from domestic dogs kept by the farmers. In the lowland Kim Thanh district of Hai Duong Province (20°56’0”N/106˚19’0”E), 57 blood samples were taken from indigenous cattle and 11 from dogs (Fig. 1A). The ethical approval was approved by the National Institute of Malariology, Parasitology and Entomology (NIMPE) on behalf of the Ministry of Health, Vietnam. Appropriate permission was obtained from the commune authorities and consent was obtained from cattle and dog owners. Blood samples from the animals were taken by experienced technicians/veterinarians.

About 2 ml of whole blood was collected from each animal into vials containing ethylenediaminetetraacetic acid (EDTA) anticoagulant (Sigma-Aldrich Co. LLC, Saint Louis, Missouri, USA), and kept on ice during transportation to the laboratory. Blood smears were stained using Giemsa (Sigma-Aldrich) and observed with light microscopy at 1,000 × magnification (Fig. 1B).

Total genomic DNA was extracted from the middle phase of 400 μl of blood after centrifugation, from those individual hosts with *Anaplasma* spp. present in their blood smears. DNA extraction was done using the GenElETM Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Amplification of a portion of the 16S rDNA gene was performed by nested PCR using first the primer pair (forward EHR1 5’ GAACGAACGCTGGCGGCAAGC 3’; and reverse EHR2 5’ AGTYCGRACCAGATAGCCGC 3’). Two microlitres of the first PCR product was used for the second, nested, PCR using primer pair EHR3 (5’ TGCATAGGAATCTACCTAGTAG 3’) and EHR4 (5’ CTAAGAATTCCGCTATCCCTCT 3’) [12]. Each PCR was done in a reaction volume of 50 μl containing 25 μl DreamTaq PCR Master Mix from Thermo Fisher Scientific, 2 μl of each primer (10 pmol/μl), 2 μl DMSO, 16.0 μl pure water, and 3 μl template (~10 ng/μl). Amplification was carried out in a MJ thermal cycler PTC-100 (MJ Research, Watertown, Massachusetts, USA), with denaturation at 94°C for 5 min, followed by 35 cycles, each of 94°C/30 sec, 52°C/30 sec (annealing), 72°C for 2 min (extension), followed 10 min/72°C (final extension). The final PCR product was 510-520 bp in length, of which a 500-501 bp portion was used for alignment for taxonomic identification.

Our 10 16S rDNA sequences of *Anaplasma* spp. were supplemented with 15 *Anaplasma* sequences from GenBank. A sequence from *Ehrlichia canis* was used as an outgroup for phylogenetic analysis. All 26 sequences were aligned in GENEDOC 2.7 (Available from: http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html). A phylogenetic tree was inferred using the maximum likelihood method in MEGA7 with the general time reversible model (GTR+G+I).
Support for each node was assessed using 1,000 bootstrap resamplings [18].

Positive Giemsa-stained blood smears showed *Anaplasma* spp. present within bovine erythrocytes, with *A. marginale* typically located near the membrane (Fig. 1B). This species was identified on the basis of 16S rDNA analysis in 5 indigenous cattle (3 in Ha Noi; 2 in Hai Duong Province) and 2 dairy cattle (Ha Noi). *Anaplasma platys* was detected in 2 indigenous cattle (1 in each province) and 1 dog (in Ha Noi Province) (Table 1). Sequences of all 7 Vietnamese samples of *A. marginale* were identical and differed by 0.2%-0.4% from the reference sequences used (strains from China and South Africa). Three other Vietnamese isolates identified as *A. platys* differed by less than 0.2% from the reference strains from Germany and the Philippines.

### Table 1. Molecular identity of *Anaplasma* spp. from indigenous and dairy cattle and domestic dog in 2 provinces in northern Vietnam

| No. | Hosts                  | Date of isolation | Geographical locality of collection | Sample code | Species identification      | GenBank No. |
|-----|------------------------|-------------------|-------------------------------------|-------------|----------------------------|-------------|
| 1   | Indigenous cattle      | 12-Dec-16         | Ba Vi district, Ha Noi              | ANA25BV     | *Anaplasma marginale*       | MH686041    |
| 2   | Indigenous cattle      | 12-Dec-16         | Ba Vi district, Ha Noi              | ANA88       | *Anaplasma marginale*       | MH686042    |
| 3   | Hybrid dairy cattle    | 05-Sep-17         | Ba Vi district, Ha Noi              | BS27        | *Anaplasma marginale*       | MH686043    |
| 4   | Hybrid dairy cattle    | 05-Sep-17         | Ba Vi district, Ha Noi              | BS255       | *Anaplasma marginale*       | MH686044    |
| 5   | Indigenous cattle      | 05-Aug-17         | Ba Vi district, Ha Noi              | BV1         | *Anaplasma marginale*       | MH686045    |
| 6   | Indigenous cattle      | 08-Aug-17         | Kim Thanh district, Hai Duong       | BVHD1       | *Anaplasma marginale*       | MH686046    |
| 7   | Indigenous cattle      | 08-Aug-17         | Kim Thanh district, Hai Duong       | BVHD2       | *Anaplasma marginale*       | MH686047    |
| 8   | Indigenous cattle      | 12-Dec-16         | Ba Vi district, Ha Noi city         | ANA111      | *Anaplasma platys*          | MH686048    |
| 9   | Indigenous cattle      | 08-Aug-17         | Kim Thanh district, Hai Duong       | BVHD4       | *Anaplasma platys*          | MH686049    |
| 10  | Domestic dog           | 10-Jan-17         | Ba Vi district, Ha Noi              | C9BV        | *Anaplasma platys*          | MH686050    |

Fig. 2. Phylogenetic tree showing taxonomic relationships of *Anaplasma marginale* detected from cattle and *A. platys* from cattle and dogs, in 2 provinces in northern Vietnam. The relationships were inferred based on 16S rDNA data (501-502 bp) from 26 sequences, including 10 from Vietnam and 15 reference sequences of *Anaplasma* species. *Ehrlichia canis* was used as an outgroup. Phylogenetic reconstruction was performed in MEGA7 using maximum likelihood analysis based on the general time-reversible model. Support for each node was indicated by 1,000 bootstrap resamplings [18]. The scale bar represents the number of substitutions per site. For each sequence, the full species names are followed up by strain abbreviations (in brackets) and the country (where they were isolated). The accession numbers are given at end of each sequence label. Ten *Anaplasma* spp. from Vietnam are marked with a solid circle. Names of hosts (cattle or dogs, in brackets) are added in the label for every sequence from Vietnam.
The inter-specific distance between _A. marginale_ and other _Anaplasma_ spp. was 4.6-5.0% (with _A. bovis_), 2.8-3.6% (with _A. platys_), and 1.0-3.6% (with _A. phagocytophilum_).

Phylogenetic analysis (Fig. 2) revealed the close relationship between the 7 _A. marginale_ sequences from Vietnam and the reference strains from China, The Philippines, Zimbabwe and South Africa (99% bootstrap support). Similarly, the 3 Vietnamese _A. platys_ sequences formed a well-supported group (90% bootstrap support) with 6 reference strains from China, Malaysia, Thailand, Germany, The Philippines and France. _Anaplasma phagocytophilum_ (KT986058, South Korea; U02521, United States) and _A. bovis_ (KY425445, Australia) formed separate branches in the tree, indicating a relatively distant relationship between _A. marginale_ and _A. platys_ (Fig. 2).

In this study, we have molecularly detected and identified _Anaplasma_ spp., including _A. marginale_ in the hybrid dairy cattle; _A. platys_ in a domestic dog; and both species in the indigenous cattle. Phylogenetic tree showed the clear topology for 2 separate clusters of _Anaplasma_ spp. of the Vietnamese samples indicating _A. marginale_ and _A. platys_, respectively. Low genetic distance within strains in each species supported the taxonomic analysis.

_A. marginale_ is specific to bovine hosts, infecting primarily lactating cattle, particularly hybrid cows, while _A. platys_ has a wider host range that includes cattle, dogs and humans [3,15]. Our discovery of _A. platys_ in cattle and domestic dogs raises considerable concern about zoonotic transmission in Vietnam, where indigenous cattle are grazed on grass fields and hybrid dairy cattle are kept in pens, fed daily by the farmers. Domestic dogs are commonly kept around farmers’ homes. This means very close contact between the host animals and the human population. It is important to plan future surveys, applying both morphological and molecular methods, for investigation of rickettsial bacteria in animals and humans in endemic areas through Vietnam.

In conclusion, our study has provided molecular species identification of _Anaplasma marginale_ in hybrid dairy cattle and indigenous cattle, and of _A. platys_ in cattle and dogs in 2 provinces in the Northern Vietnam. The presence of _A. platys_ in cattle and dogs may constitute a potential zoonotic risk to humans. The systematic investigation of _Anaplasma_ spp. and members of the Anaplasmataceae in animal hosts and humans is needed across Vietnam.

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**CONFLICT OF INTEREST**

The authors declare no conflicts of interest in submission/publication of the data in this article.

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