PROCEEDING

international seminar

STRATEGY TO MANAGE BIO-ECO-HEALTH SYSTEM FOR STABILIZING ANIMAL HEALTH & PRODUCTIVITY TO SUPPORT PUBLIC HEALTH

Surabaya-Indonesia, 19-20 June 2012
JW Marriott Hotel Surabaya

EDITORS:
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I-MHERE SUB-COMPONENT B.2.C PERFORMANCE BASED CONTRACT

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MORPHOSPESIES AND PHYLOGENETIC TREE ANALYSES OF LEUCOCYTOZOON CAULLERYI FROM CHICKENS LEUCOCYTOZOONOSIS CASES IN PASURUAN, EAST JAVA

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ABSTRACT

The current taxonomy of leucocytozoids is based on the morphology of blood stages of the parasites and on limited information about their specificity. Recent molecular studies have revealed a remarkable genetic diversity of leucocytozoids, indicating that their taxonomic diversity may be greater than in the current classifications. We addressed this issue using morphological data and phylogenetic analysis of the cytochrome b gene of 4 positively identified species of Chickens Leucocytozoonosis in Pasuruan, East Java. Based on the current taxonomy, Leucocytozoon caulleryi is the sole species of leucocytozoids parasitizing chicken. We investigated the morphology of blood stages of leucocytozoids of 4 haplotypes and concluded that these parasites can be readily distinguished due to length of the cytoplasmic processes of their host cells; therefore, they do represent distinct morphospecies. Morphology of the cytoplasmic processes of host cells warrants more attention in the taxonomy of Leucocytozoon species. Leucocytozoon caulleryi indeed is a species group that currently can be separated from other Leucocytozoon by morphospesies analysis. It is can be concluded by phylogenetic tree analysis that 4 Leucocytozoon caulleryi from Pasuruan closely related strain.

Keywords: *Leucocytozoon caulleryi,* morphospesies, phylogenetic tree, cytochrome b gene

INTRODUCTION

Leucocytozoon in Pasuruan, East Java is one of the parasites that can attack the chickens throughout the year although the frequency is not fixed, and in certain areas are endemic. Services for Pasuruan Animal Husbandry has never reported a poultry disease caused by Leucocytozoon although the disease is highly detrimental to farmers so that there is no government intervention associated with the control and eradication of this disease. Hence the publication of this research is expected to provide input to the government to Leucocytozoonosis be one of the diseases included in control programs.

Leucocytozoon spp morphological identification is often not determined until the determination of species due to the occurrence of morphological variation so that the difficulties in characterizing the morphology of the parasite. Phenotypic characters can overlap among species and the real changes that can occur among isolates of the same species. With advances in biomolecular engineering, the morphological diversity can be searched directly cause the gene level, the material responsible for the occurrence of morphological variation. The use of DNA sequences in population genetic studies has grown rapidly, providing more in-depth information about the evolution and phylogenetic relationships. Series of genetic information contained in mitochondrial DNA has been reported to describe the characteristics of a population, phylogenetic and reconstruct the evolutionary history (Kvist, 2000). The use of phylogenetic tree can be useful to know the causative agent relationship in Leucocytozoonosis cases that happened in Pasuruan.

MATERIALS AND METHODS

Study Sites and Blood Samples

Blood samples were collected in Pasuruan, East Java from September to December 2011, as described by Sehgal *et al.* (2006). Blood was taken by venipuncture of the medial metatarsal vein. Extraction of DNA and PCR and sequencing was done during our recent study (Sehgal *et al.*, 2006). Blood smears were fixed in methanol and stained with Giemsa, as described by Valkiunas (2005). Positive blood samples, as determined by PCR-based analysis (Sehgal *et al.*, 2006), from 10 chickens that have sign of Leucozoonosis were used for investigations of the morphology of leucocytozoids and their host cells. In morphological analysis, we used only a good-quality blood film, which is essential for taxonomic studies (Valkiunas *et al.*, 2008). Sehgal *et al.* (2006) described details of the study sites and collection of the material.

Morphological Analysis

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution, GmBH, Munster, Germany) was used to examine blood films, prepare illustrations, and to take measurements. Intensity of infection was estimated as a percentage by actual counting of the number of parasites per 1,000 red blood cells, or per 10,000 red blood cells if infections were light, i.e., ,0.1%, as recommended by Valkiunas *et al.* (2010). Blood films with intensity of Leucocytozoon spp. Intensity of parasitemia in the investigated chickens was light (,0.01%), so this sample was not used for morphometric analysis. The morphometric features studied (Fig.1) were those defined by Valkiunas (2005).

Phylogenetic Analysis

The phylogenetic analysis was based on sequences (500 bp) of the cyt b gene PCR product compare to sequences of GenBank. Phylogenetic analyses using maximum parsimony techniques were conducted using PAUP*4.0b10 (Swofford, 2002). Searches used the bootstrap search option, with 1,000 stepwise addition replicates, using the TBR branch-swapping algorithm. In addition, we performed distance analyses using the Kimura 2-parameter distance model, and taxa were joined using neighbor-joining analysis (Perkins and Schall, 2002; Sato *et al.*, 2007). Simple consensus trees were constructed to summarize the results; both maximum-parsimony and neighbor-joining analyses resulted in trees with identical topologies. The sequence divergence between the different lineages (Fig. 2) was calculated with the use of a Jukes-Cantor model of substitution, with all substitution weighted equally, implemented in the program CLUSTALW (Kumar *et al.*, 2004).

RESULTS AND DISCUSSION

Morphological Analysis

There were no significant differences in all morphometric features of Leucocytozoon sp. gametocytes and their host cells in different individual hosts belonging to the same species of chicken, so we pooled morphometric data for parasite measurements in different individual hosts belonging to the same chicken species. These data are given in Figure 1.

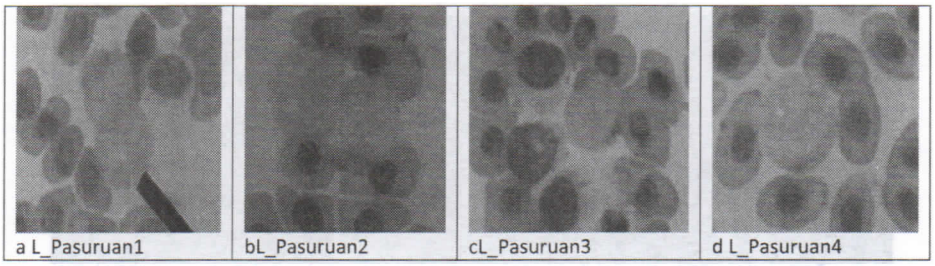

Figure 1. a-d. Gametocytes of Leucocytozoon caulleryi in fusiform host cells from the blood of Chickens

Phylogenetic Analysis

Parasites from 4 Pasuruan chickens cluster together, and form distinct on clades in the phylogenetic tree (Fig. 2). Sequence divergence between 4 positively identified as Leucocytozoon caulleryi. **Scores**

Table 1. The sequence divergence (in percentage) between mitochondrial cytochrome b lineages of positively identified species of Leucocytozoon caulleryi GenBank, L. caulleryi Pasuruan and Plasmodium.juxtranucleare.

SeqA	Name	Length	SeqB	Name	Length	Score
1	Leucocytozoon_ caulleryi_AB302215	493	2	Leucocytozoon_ Pasuruan1	493	99.0
1	Leucocytozoon_ caulleryi_AB302215	493	3	Leucocytozoon_ Pasuruan2	493	100.0
1	Leucocytozoon_ caulleryi_AB302215	493	4	Leucocytozoon_ Pasuruan3	493	97.0
1	Leucocytozoon_ caulleryi_AB302215	493	5	Leucocytozoon_ Pasuruan4	493	99.0
1	Leucocytozoon_ caulleryi_AB302215	493	6	Plasmodium_ juxtanucleare_AB302893	478	88.0

We encourage using morphological analysis of the cytoplasmic processes during description of Leucocytozoon spp. Such processes develop only in some species of Leucocytozoon; the function of the processes remains unknown. It is important to note that the character presence or absence of the cytoplasmic processes can hardly be applied in the taxonomy of leucocytozoids, on either the level of genera or subgenera, because lineages of parasites possessing the cytoplasmic processes of Leucocytozoon caulleryi are paraphyletic in the phylogenetic analysis (Fig. 2). This conclusion is in accord with former taxonomic conclusions which are based on morphology and life histories of leucocytozoids (Fallis *et al.*, 1974). The present study shows that morphology of the cytoplasmic processes of host cells warrants more attention in the taxonomy of Leucocytozoon species. Detailed analysis of morphology of the processes has not been applied in taxonomy of these parasites, so far. It is worth noting that development of the fusiform processes of host cells is induced by developing gametocytes Because the development of the fusiform processes is parasite species-specific (Valkiunas, 2005), it should be genetically determined and might reflect evolutionary adaptations and, thus, be applied in Leucocytozoon taxonomy at the species level.

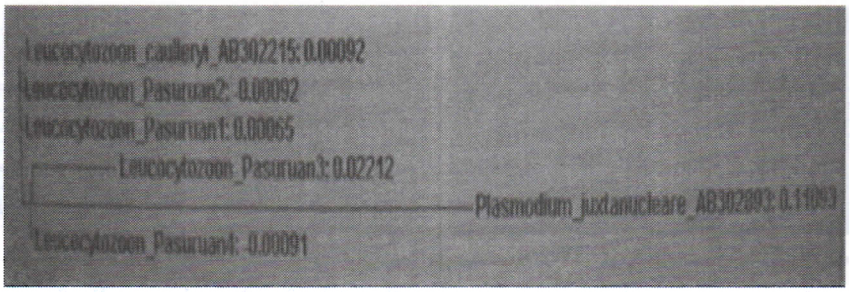

Figure 2. Maximum-parsimony phylogeny of 4 mitochondrial cytochrome b lineages of Leucocytozoon species. Lineages of 1 species of avian Plasmodium juxtranucleare from GenBank were used as an outgroup. GenBank accession number of the L. caulleryi sequences for controlling analysis. The names of Leucocytozoon species, which develop in fusiform host cells, are given in bold. The branch lengths are drawn proportionally to the amount of change.

We suggest the validation of these names by BLAST software and use them to identify morphologically similar parasites (Figs. 1) of closely related lineages (Fig. 2). This conclusion is in accord with the hypothesis of Omori *et al.*,(2008) that L. caulleryi might represent morphologically similar taxa comprising a species complex. We agree, and suggest identifying parasites of this complex as a group of L. caulleryi species by using Basic Local Alignment Search Tool (BLAST).

It is probable that the criterion of a genetic difference in cyt b gene eflects interspecific divergence in many groups of avian haemosporidians, and so can be used for better understanding of phylogenetic trees based on this gene. It should be noted, however, that genetic divergence in the cyt b gene between some readily distinguishable morphospecies of avian haemosporidian parasites is some readily distinguishable morphospecies (Hellgren *et al.*, 2007; Valkiunas *et al.*, 2009). Thus, the molecular criterion of sequence divergence in the cyt b gene for identification of haemosporidian species should be developed and applied carefully, preferably by linking molecular and microscopical data. To fully accept this criterion, additional information about genetic distances between lineages of positively identified species of haemosporidians is needed (Table 1). The conclusion on phylogenetic tree analysis that 4 Leucocytozoon caulleryi in Chicken Leucocytozoonosis from Pasuruan, East Java is one strain of spesies Leucocytozoon caulleryi.

It is important to note that PCR detects very small numbers of unknown sporozoites in the peripheral circulation (Valkiunas *et al.*, 2009), so detection of lineages of hemosporidians in vertebrate hosts should be carefully considered in ecological and evolutionary biology studies. To be accepted as the lineages of successfully developing species of hemosporidians, such PCR-based information should be supported with the detection of blood stages of the parasites. We thus emphasize an urgent need for the synthesis of information provided by tools of traditional parasitology and molecular biology in studies of haemosporidians, particularly Leucocytozoon caulleryi in Indonesia.

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2.	Protective Immunity of *Eimeriaacervulina* Oocysts Protein Against Intestinal Coccidiosis	2020
3.	Reproductive characteristic of a precocious line of E. tenella sporozoite as material bioactive in embyionating eggs and the implications of the findings appraised	2014
4.	Vaksininasi Protein Ekskretori-Sekretori Toxoplasma gondii Hasil Biakan in vivo Membangkitkan Respons Imun Non Protektif	2013
5.	Pengaruh ekstrak sambiloto (Andrographis paniculata) terhadap produksi ookista dan skor lesi sekum ayam yang diinfeksi Eimeria tenella	2016

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6.	The conformity of diagnostic test between burrow ink test method with skin scrapping method of scabies in rabbit (Lepus domesticus)	2018
7.	The Endogenous Development of Eimeria tenella in Chickens Injected Subcutaneously with Oocysts Protein as Initially Study of Development of Cecal Coccidiosis Killed Vaccine.	2020
8.	Potency of Attenuated Eimeria tenella in Protective Immunity Induction on Homologous and Heterologous Challenges	2016
9.	Morphospesies and phylogenetic tree analyses of Leucocytozoon caulleryi from chicken leucocytozoonosis cases in Pasuruan, East Java.	2012

Adapun penelitian tersebut layak dilakukan, meskipun belum ada ***Uji Etical Clearence*** karena menggunakan hewan coba yang minimal dan menghasilkan output yang sangat baik.

Demikian surat kerangan ini kami buat untuk dapat dipergunakan sebagai persyaratan pengusulan Jabatan Fungsional **Guru Besar**

Surabaya, 8 Agustus 2022

Dekan,

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