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A Development of Homolog Sequence of *Eimeria tenella* Partial Genome as a Probe for Molecular Diagnosis of Coccidiosis

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Abstract

The goal of the research was to develop a homolog sequence of *Eimeria tenella* partial genome as a molecular probe for diagnose coccidiosis using dot blot method. A probe of homolog sequence of *E.tenella* partial genome and a non radioactive label, dig-11-dUTP, were used for this research. Four concentrations of molecular probe labeled with dig-11-dUTP, namely, 158,33 pg/µl, 52,25 pg/µl, 15,83 pg/µl and 5,225 pg/µl were tested to detect 0,6551 µg DNA target. The procedure of labeling and hybridization detection between DNA target with the molecular probe labeled with dig-11-dUTP were carried out with Digh high prime DNA labeling and detection starter Kit I. The conclusion of the research was that 52,25 pg/µl molecular probe or more which its sequence **GGCA CAGTATCCTCCTTCAGGGCAGGG CTCGCACTGGTCAAA CGCGG TAC CATT** could detect DNA target by dot blot method.

Keywords: coccidiosis, *E. tenella* genome, molecular probe, dot blot hybridization

Introduction

One of the harmful of chicken diseases is coccidiosis. According to Groves (1986), there are nine species of Eimeria as causative agents of chickens coccidiosis. The most pathogene of Eimeria species is *E. tenella* (Reid *et al*., 1984; Kaufmann, 1996, Robert and Janovy, 2000). The life cycle of the Eimeria consist of three phases, namely, sporogony, schizogony and gametogony (Soulsby, 1982; Kaufmann, 1996). According to Calnek (1991) and Soulsby (1982), The schizogony II phase is the most destructive for the chicken. The diseases may be diagnosed based on the clinical symptoms, oocysts finding in the faeces and on the postmortem examination, it may be diagnosed based on Eimeria stadia finding in the chicken intestine (Soulsby, 1982; Morgan and Hawkins, 1995; Bowman, 2003). Recently, many molecular methods have been developed to diagnose coccidiosis such as: DNA *(deoxyribonucleic acid)* detection and identification (MacPherson and Gajadhar, 1993; Barker, 1990; Reischsl *et al.,* 2003). According to Shirley (2000), the genom of *E. tenella* is 60 Mbp in the ca. 14 chromosom, whereas according to Anonim (2001) the genom size is 70 Mbp. In Indonesia, the molecular diagnose for coccidiosis have not developed yet. Sumartono *et al.* (2004) sequenced a homolog band of partial genom from five Indonesian isolates.

The aim of the research is to study the sequenced band as a probe to molecular diagnose of coccidiosis using dot blot hybridization (Salehzada dan Taha , 1992).

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Materials and Methods

The main materials was $3,54.10^6$ oocystes isolat Yogya, an oligonukleotida : **GGCACAGTATCCTCCTTCAGGGCAGGGCT CGCACTGGTCAAACGCGGTAC CATT** (Sumartono *et al*., 2004), and Dig. Control test

strip, Dig high prime DNA labeling and detection starter Kit I (Roche, Germany).

Isolation of *E. tenella* genom

The genom was isolated from $3{,}54.10^6$ *E. tenella* oocystes of Yogya isolate. The isolation was carried out using the modification of MacPerson and Gajadhar methode (Sumartono *et al.,* 2004).

Preparation of homolog sequence as a probe candidate

The probe candidate used in the research was from Sumartono *et al.,* (2004). Sequence analyses was done either manually or using *gene analyzer*. The probe candidate was chosen by comparing sequence manually analysed to sequence using gene analyzer.

Probe labeling

Probe labeling was done with a non radioaktif reporter, digoxigenin-11-dUTP. A number of 2 µl probe candidate (1 g) and 14 ml aquadest were filled in a 1.5 ml eppendorf tube. After boiling for 10 minute, the tube was subsequently chilled on ice. Then, a number of 4 µl dig high prime (consist of Klenow enzyme, labeling grade, dig-11-dUTP, alkaly labil, dATP, dCTP, dGTP, dTTP and reaction buffer optimized with 50% gliserol) was added. Before incubation at 37°C for overnight, the solution was condensed for a moment by centrifugation. For stopping the reaction, a number of 2 µl 0,2 M EDTA (pH 8) was added to the tube.

Quantification of labeled probe

Quantification of labeled probe bounded by antibody anti dig-11-dUTP was estimated using dig DNA labeling and detection Kit and DIG quantification teststrips (Boehringer, Germany). Principally, the quantification was done by comparing hybridization of labeled probe test to a controle teststrip contained a known concentration. Solution preparation, dilution of labeled probe, teststrip preparation and detection of labeled probe were carried out according to the procedure of the Kits.

Conception of diagnose model for an application of dot blot hybridization

A teststrip model was designed for an application of dot blot hybridization. The teststrip consisted of 5 compartment (compartment 1: Blank, compartment 2: A, compartment 3: B, compartment 4: C, and Compartment 5: D)

Optimalisation of labeled probe use

The procedure consisted of teststrip preparation, series dilution of labeled probe in hybridization solution, hybridization and immunology detection.

For teststrip preparation, a number of 8 teststrips was divided into 4 groups (I-IV), each group consisted of 2 teststrips. A number of 10 µl *E. tenella* DNA of Yogya isolat (1 µl = 0.6551 µg DNA) was boiled at 100°C for 10 min and, then, it was chilled on ice. On compartment B of teststrips, a number of 1 µl *E. tenella* DNA was dotted and 1 µl ddH_20 was dotted on compartment C. After washing in 2x SSC for a moment, those teststripts were baked at 80°C for 2 h. Teststripts can be stored in refrigerator (2 - 8°C).

Preparation of series dilution of labeled probe in hybridization solution was carried out in three steps. The first step was preparation of hybridization of solution, the second step was preparation of a series dilution of labeled probe and the third step was preparation of a series dilution of labeled probe in hybridization solution.

Hybridization solution was made by diluting Dig. Easy Hyb. granule in 64 ml ddH_20 and it was stirred for 5 min at 37°C. For making a series dilution of labeled probe in DNA dilution buffer, it was done by the same procedure with the procedure of

dilution of the Kit. Series dilution of labeled probe in hybridation solution were made by mixing the two solutions. Dig. Easy Hyb. solution was divided into 4 aliquot , each consisted of 1 ml. A number of 2 µl of every serie dilution of labeled probe was added into the aliquot so there were 4 aliquots with different concentration of labeled probe. Those aliquots were stored at -15 to - 25°C.

Before making hybridization between labeled probe to DNA target, the temperature of hybridization was estimated and Dig.Easy Hyb. solution was heated at hybridization temperature. The estimation was carried out according to Keller and Manak (1989). The prepared teststrips was filled in the plastic sachets, each consisted of 2 teststripts according to their code. A number of 1 ml of heated Dig. Easy Hyb. solution was added in the sachets. Those sachets were agitated moderately for 30 min. After pippeting out solution in the sachets, the sachet was filled in 1 ml of serie dilution of labeled probe in Dig. Easy Hyb. solution. Those sachets was reagitated moderately for 4 h. The solution in the sachet was repippeted out and, then, it was washed 2 x with the 50°C of washing solution. The washing was done by moderate agitation for 5 min.

Hybridization between labeled probe to DNA target was detected by using an antibody-antiDig-AP (AP: alkaline phosphatase). A series dilution of labeled DNA control were made by the same procedure with dilution procedure of labeled probe candidate. A number of 1 µl control solution was dotted on compartment A of the teststrip according its concentration, and as a negative control, on compartment C was dotted with ddH_20. Detection procedure of the hybrid was carried out by the same procedure with procedure of the Kit.

The data was analysed descriptively.

Results and Discussion

On OD260, the genome of *E. tenella* obtained was 0.262, Its means that the genome in the sample was: 0.262 x 50 x 50 (value dilution) = 655, or the genome concentration in the elution (50 µl) was: 655 x 0.05 = 32.75 µg / 50 µl or 0.655 µg /µl.

Probe candidate from Sumartono *et al.* (2004) analysed with two different method was presented on Table 1.

Tabel 1 showed that, based on the longer of the probe, the two sequence maybe to be a probe because, these sequences were not too longer to be a probe (77 nt vs 54 nt). According to Keller and Manak (1989), the good probe contains 50 bases, whereas, the good probe may be longer, 100-300 bases (Leitch *at al.*, 1994). The GC ratio of two sequence (50.6% vs 57%) was higher than GC ratio according to Keller and Manak (1989) (40% - 60%). Nevertheless, the number of the same nucleotide sequence was fewer in the sequence analysed by gene analyzer than in sequence analysed manually (3 (2 (GGG), 1 (AAA)) vs 5 (3 (GGG), 2 (AAA)). So, the probe candidate used in the research was **GGCACAGTATCCTCCTTCAGGGC AGGGCTCGCACTGGTCAAACGCGGT ACCATT.**

Table 1. Probe candidate analysed manually and using gene analyzer.

Manual Anlyses (Result. HBXII/1)	GenetyxMax Analyses
AGGGCAGGGCTCGCACTGGTCAAAG	GGCACAGTATCCTCCTTCAGGGCAG
GCGGTACCATTTGGGTCCTTTCGTAT	GGCTCGCACTGGTCAAACGCGGTAC
GGTGCCTAAATTCATGACCACACTA TT	CATT

Labeling of molecular probe candidate for coccidiosis diagnose presented on the Figure 1. Concentration of probe candidate in stock was 825 µg / 1500 µl or 0.55 µg/µl. The number of labeled probe candidate was 2 µl or 1.1 µg (1100 ng). By incubation for 20 h in 20 µl solution, its means that labeled probe candidate was 38/100 x 1100 pg = 418 ng in 20 µl solution or 20.9 ng/µl. For making dilution of the labeled candidate probe, 1 µl the labeled probe was diluted to 40 µl, its means that in 1 µl of this solution contained 0.5225 ng labeled probe. In the 10 µl dilution A contained 5.225 ng labeled probe candidate, the solution was diluted with DNA dilution buffer into 33 µl. For dotting, it was used 1 µl of dilution A or 158.33 pg, so its means that for dilution B was 52.25 pg, dilution C was 15.83 pg, dilution D was 5.225 pg and dilution E was 1.583 pg. Figure 1 showed that on teststrip control, the antibody anti dig-11-dUTP could detect the labeled probe candidate in all compartments (3 pg to 300 pg), whereas, on probe candidate teststrip the antibody only detected the probe 5.225 to 158.33 pg. Nevertheless, Its means that the smallest probe candidate detected was > 3 pg. According to the protocol of the Kit, the labeled probe can be used for the next procedure (hybridization).

The hybridization temperature was estimated according to Keller dan Manak formula (1989).

T_m = 49.82 + 0.41 (% G + C) – (600 /l) (l : number of probe nucleotide)

T_{opt} : T_m – 20 to 25°C

In the research :

% (G + C) probe candidate was 59.26, and l = 54, T_m = 49.82 + 0.41 (59.26) – (600 / 54) = 49.82 + 24.30 – 11.11 = 63.01. So, for hybridization, the optimal temperature was 38.01°C – 43.01°C or 40.51°C.

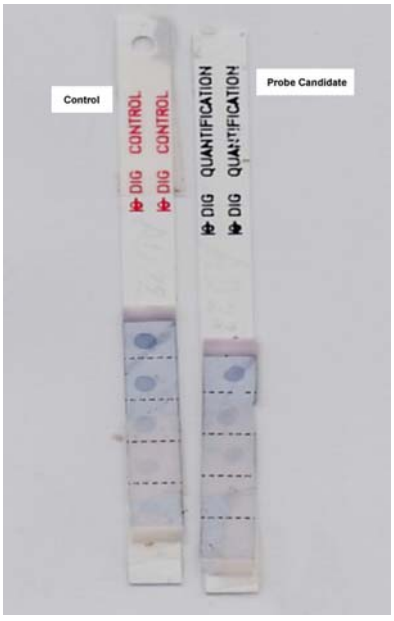

Figure 1. Labeling of molecular probe candidate with non radioaktif dig -11-dUTP.

The optimalisation of labeled probe use for coccidiosis diagnose was presented on Figure 2.

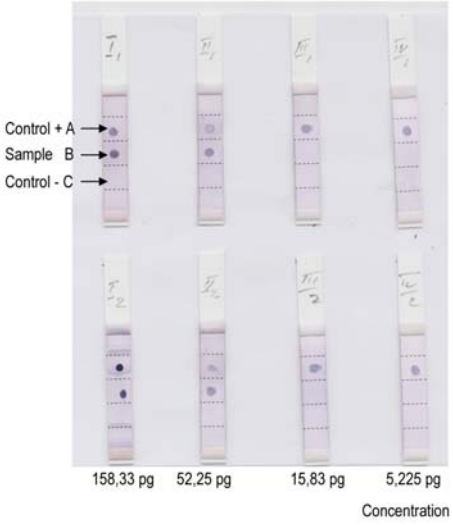

Figure 2. Result of the optimalisation of labeled probe use.

On Figure 2 there were 2 lines teststrips, namely, line I_1 –IV_1 and line I_2 – IV_2. Line I_2-IV_2 was a repetion of teststrip I_1-IV_1. On the teststrip showed that the dot was coloured. The formation of the coloured dot showed that a reaction between antibody-antidig-11-dUTP conjugated with an enzyme, *alkaline phosphatase* (AP) to the substrat NBT/BCIP. The formation of coloured dot on compartment control positive (A) on all teststrip showed that the test was well procedure, its means that antibody-antidig-11-dUTP could detect dig-11-dUTP bounded by the probe. The formation of coloured dot on compartmens (B) of teststrip (I_1, II_1, I_2 and II_2) showed that the probe could detect *E. tenella* DNA fixed on the teststrip (0.6551 µg). On the same compartment (B), on teststrips III_1, IV_1, $IIII_2$ and IV_2, the coloured dot were not appear because of lower concentration of labeled probe. The On compartment C of the teststrips, the coloured dot, also, was not appears because no DNA target on the compartment (control negative), and labeled probe washed away at the time of detection procedures. That means that the probe candidate which its sequence was **GGCACAGTATCCTCCTTCAGGGCAGGGCTCGCACTGGTCAAACGCGGTACCATT** and on minimal concentrationl 52.25 pg could be used to detect 0.6551 µg *E. tenella* DNA by dot blot application.

The research concluded that a sequence, **GGCACAGTATCCTCCTTCAGGGCAGGGCTCGCACTGGTCAAACGCGGTACCATT**, of *E. tenella* partial genome could be used to a moleculer probe to coccidioses diagnose by dot blot application on minimal concentration 52.25 pg.

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