Identification of animal derivatives contained in commercial chicken feeds using multiplex-PCR

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Abstract. Availability of commercial feeds for chicken force farmers to use them due to the practicality than hand formulated feed. Cannibalism and spread of infectious disease may be occurred by feed without knowing its contents. Therefore, the aim of this study was to determine species contained in commercial feeds for chicken using multiplex-PCR mitochondrial DNA Cytochrome b gene. This study used five commercial feeds for chicken produced by five different companies as samples. They were namely PK1, PK2, PK3, PK4, and PK5. Hand-made meat meals from goat, chicken, bovine, and pig were used as positive controls. Genomic DNA was extracted from those samples, and they were used as DNA template for PCR. The PCR products were then visualized using 2% agarose gels under the UV light. The results showed that some samples were positively containing animal derivatives. The PK1 sample contained chicken, bovine, and pig which are respectively indicated by 227, 274, and 398 bp of DNA bands. Moreover, PK4 and PK5 samples contained bovine species. On the other hand, PK2 and PK3 samples did not contain animal derivatives, especially four species used as markers in this study. It can be concluded that animal derivatives have been detected in three commercial feeds for chicken using the multiplex-PCR technique. This study recommended avoiding a commercial feed for chicken since it contained chicken derivatives to prevent cannibalism.

1. Introduction
Feedstuff is feed ingredient which is able to either partially or completely be eaten, digested, and absorbed by livestock and it does not cause animal health problem [1]. The availability of protein in feed plays an important role for animal growth. Most of commercial feeds use animal protein resources as feedstuff due to their positive effect to animal performance [2]. Fish meal, blood meal, feather meal, and meat bone meal are animal protein resources which are widely used in commercial feed in the form of mash, pellet, and crumble [3,4].

Commercial feeds which are marketed in Indonesia must be registered and have feed registration number issued by Ministry of Agriculture through Director General of Livestock and Animal Health [5]. Feed registration number is a certificate containing letters and numbers explaining the identity of the feed which functions as a sign of the feed validity to be distributed. Commercial feed is also need
certificate of feed quality and safety which is issued by accredited laboratory. Moreover, European Community (EC) number 1777/2002 to EC No. 1069/2009 states prohibition of feed containing protein derived from the same species to avoid cannibalism and the risk of spreading the disease through feed and animal products [6]. Identification of animal derivatives in feed cannot be performed by bare eyes [7]. Therefore, DNA barcoding will be powerful technique to evaluate species contained in feed [8].

Conventional polymerase chain reaction (PCR) is still the best choice to identify species in the product due to efficiency reasons [9]. Multiplex PCR, a conventional DNA barcoding, is quick, cheap and simple simultaneous PCR technique for identification species in food and feed [7,8]. Mitochondrial DNA (mt-DNA) becomes main targeting region in multiplex PCR for species identification such as cytochrome b, 12S rRNA, COI gene, and D-loop [7,10–12]. Currently, identification animal derivatives in commercial feed sold in Indonesia has not been reported yet. Therefore, the objective of this study was to identify animal derivatives contained in commercial chicken feeds using multiplex-PCR.

2. Materials and methods

2.1. Sample collection
Commercial feed samples for chicken were collected from feed shop around Surakarta city. Feed shop selling commercial feed for chicken produced by company was selected. A total of five commercial chicken feeds from different company were used in this study labelled as PK1, PK2, PK3, PK4, and PK5. Nutrient contents and feedstuffs were recorded in accordance to the information written in the package.

2.2. Producing meat meal
Meat meal was produced as positive control. Meats used as meat meal material were beef (bovine), chevon, pork, and chicken. A total of 250 g each meat was washed and thinly sliced (3 mm). Meats were then dried at 60°C for 24 hours. Furthermore, dried meats were separately grinded finely [13].

2.3. The DNA extraction
A total of nine DNA genomes was successfully extracted from five chicken commercial feeds and four positive controls. Previously, 25 mg dried samples were diluted with 20 µL ddH2O. The DNA genome was then extracted by following procedure of Genomic DNA Mini Kit for animal tissue (Genaid Biotech Ltd., Taiwan).

2.4. Multiplex-PCR
Amplification of DNA was conducted by targeting mt-DNA Cytochrome b previously reported by Matsunaga et al. [10]. Primers used in this study are presented in table 1.

| Species   | Oligonucleotide primer (5’ to 3’) | PCR product size (bp) |
|-----------|----------------------------------|-----------------------|
| Universal forward primer            | GAC CTC CCA GCT CCA TCA ACA ATC TCA TCT |                     |
| Bovine     | CTA GAA AAG TGT AAG ACC CGT AAT ATA AG  | 274 bp                |
| Chicken    | AAG ATA CAG ATG AAG AAG AAT GAG GCG  | 227 bp                |
| Goat       | CTC GAC AAA TGT GAG TTA CAG AGG GA  | 157 bp                |
| Porcine    | GCT GAT AGT AGA TTT GTG ATG ACC GTA | 398 bp                |

A total of 25 µL reaction containing 12.5 µL PCR master mix (MyTaq™ HS Red Mix Bioline, UK), 1 µL each primer, 1 µL DNA template, and 6.5 µL ddH2O was conducted in this study. The PCR program was initially started by pre-denaturation at 95°C for 3 minutes and followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds.
The PCR program was terminated by final extension at 72°C for 3 minutes. Moreover, electrophoresis agarose gel (2%) was conducted at 100 volt for 30 minutes to check PCR products and then visualized under the UV light (Glite UV Gel Doc System, Pacific Image Electronic Co., Ltd., New Taipei City, Taiwan). A 100 bp marker ladder was used as DNA band standard size.

3. Results and discussion
Animal derivatives were detected in three out of five commercial chicken feed samples. The PK1 sample contained chicken, bovine, and pig-originated materials that were respectively indicated by 227, 274, and 398 bp of DNA bands (figure 1). The PK4 and PK5 samples contained bovine-originated materials. Meanwhile, animal derivatives were not detected in PK2 and PK3 samples in regard to four species used as markers in this study.

![Figure 1.](image)

Figure 1. The result of multiplex PCR analysis using Cytochrome b gene. M is 100 bp marker ladder; TB is positive control for porcine; TS is positive control for bovine; TA is positive control for chicken; TK is positive control for goat; PK1 to PK5 are commercial poultry feed samples.

Five commercial feed used as sample in this study was produced by big feed company in Indonesia. Of these, three companies stated that animal source ingredient (meat bone meal) was used as feedstuff. A company did not inform feed ingredient in the label. Moreover, a company declared that no animal source ingredient used in the formulation (table 2). Animal derivatives were detected in three commercial feed. They were PK1 containing chicken, bovine, and porcine; PK4 containing bovine; and PK5 containing bovine species. Two companies using meat bone meal in their formulas were not confirmed that their product containing animal derivatives in this study. It might be due to different species of meat bone meal origin used and high thermal treatment during process [7].

| Sample ID | Animal source ingredient labelled | Result |
|-----------|----------------------------------|--------|
| PK1       | Meat bone meal (MBM)             | -      |
| PK2       | MBM                              | -      |
| PK3       | MBM                              | -      |
| PK4       | No information                   | -      |
| PK5       | No animal source ingredient      | -      |
|           | Goat                             | 0      |
|           | Chicken                          | 20     |
|           | Bovine                           | 60     |
|           | Porcine                          | 20     |

(+ ) indicates sample containing animal derivative (- ) indicates no animal derivatives detected.
Bovine derivative was found in the commercial samples that did not provide feed ingredient in the package. A commercial feed sample (PK5) was mislabeled declaring that no animal source ingredient, however it was found containing bovine derivative. PK1 indicated containing chicken derivative should not be fed to poultry to prevent cannibalism. Cannibalism can trigger transmissible spongiform encephalopathies (TSE), scrapie, Creutzfeldt Jakob disease (CJD), and bovine spongiform encephalopathy (BSE) diseases in livestock may be due to consume prion protein originated from same species contained in feed [14,15]. This result suggested that the Indonesian regulation regarding feed ingredient is not implemented properly since a commercial chicken feed containing same species. Periodic inspection should be carried out by the government body to ensure feed safety marketed in Indonesia.

4. Conclusions
It can be concluded that animal derivatives have been detected in three commercial feeds for chicken using the multiplex-PCR technique. A commercial feed containing chicken derivatives should not be fed to poultry to prevent cannibalism and spread disease through feed.

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