Multiplex PCR assay for animal species identification in meat bone meal

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Abstract. The authenticity of meat bone meal (MBM) in animal feed is needed to be considered due to inappropriate feed ingredients for certain types of livestock may cause various diseases. This work was aimed to identify animal species in MBM using multiplex PCR assay with 12S rRNA gene as a target region. Total of eight DNA samples were extracted from three species (chicken, bovine, and porcine) which are used as positive controls and three MBM samples. The MBM samples were collected from three different importers. Additionally, a multiplex PCR assay has been performed to identify animal species in MBM. Multiplex PCR of 12S rRNA gene was designed to detect bovine, chicken, and porcine in MBM samples. The PCR products was visualized using 2% agarose gels under the UV light. The results showed that multiplex-PCR of 12S rRNA gene was able to identify bovine which is indicated by 155 bp of DNA band. Chicken and porcine were not found in MBM samples. Meat bone meal samples used in this study might be used to be a feedstuff for poultry, porcine, and other non-ruminants. In conclusion, Multiplex-PCR using mt-DNA 12S rRNA was effective and accurate technique to identify species contained in MBM samples.

1. Introduction
One of major concern in control of feed safety and feed quality assurance system is to evaluate animal origin contained in feedstuffs such as meat bone meal (MBM) [1]. Meat bone meal is an essential feedstuff in poultry and pig industries due to high protein content to fulfill nutrient needs and its price is also more competitive compare to other animal protein sources [2,3]. Most of MBMs are derivative products from cattle slaughterhouse and beef processing, therefore, their compositions could be varied depend on method and part of cattle body used when they are processed to be MBM [4]. The originality of MBM in animal feed have to be taken into account in determining of appropriate feedstuffs for specific species. Inappropriate feed formulation for some animal needs would trigger financial loss. Since 2002, European Union has repealed European Council (EC) No. 1774/2002 to EC No. 1069/2009 as regards animal byproducts and derived products not intended for human consumption. This regulation also prohibits the use of animal byproducts and derivative products for animal feed from the same species [5].

Three world level organization, World Health Organization (WHO), The World Organization for Animal Health (OIE), and Food and Agriculture Organization of the United Nations (FAO), have
officially released prohibition of feed containing ruminant tissues for ruminants in 1996 [6]. This regulation is implemented to prevent spread of infectious disease like Bovine Spongiform Encephalopathy (BSE) or commonly known as mad cow disease. Johnson et al. [7] reported that BSE can infect within cattle population and other animals through feed chain contaminated by an unusual transmissible agent which is called as a prion. Public pay much attention to BSE disease since it can transmit to human body, therefore good practice of feed quality control can be appropriate strategy to prevent the possibility of BSE agent infecting human being.

Identification of animal species in feedstuffs could be significant step as an implementation of regulation to prevent transmission of BSE and cannibalism. Microscopic analysis could be first option to detect and to characterize processed animal proteins (PAPs) in feedstuffs. However, this method relatively needs more time and microscope expertise [8]. Alternatively, determination of species could qualitatively be performed on the basis of DNA analysis through Polymerase Chain Reaction (PCR) [9,10]. Multiplex-PCR, a molecular technique developed to amplify more than one DNA targets in single PCR reaction, is known as a sensitive, specific, accurate, easy and quick method to identify species contained in MBM [8,11]. In addition, Mitochondrial DNA (mt-DNA) 12S rRNA gene could be target site for Multiplex-PCR due to its characteristics. This region can still be amplified through PCR from rendered animal byproducts as DNA template including MBM [10,12]. Therefore, this study aim was to investigate animal species in MBM collected from importers using mt-DNA 12S rRNA gene as targets by Multiplex-PCR assay.

2. Material and methods

2.1. Meat bone meal preparation
This study was initiated by taking a look at animal feedmills using MBM in the Surakarta and surrounding areas. Meat bone meal samples using in this study were selected based on exporter country of origin. Fortunately, there were three countries of origin of MBM and they were labelled as M1, M2, and M3, respectively. In addition, MBM samples were replicated and respectively labelled as M1A, M2A, and M3A. Beef (S), pork (B), and chicken (A) were collected from traditional market located in Surakarta city. Those samples were than processed to be meal in accordance to procedure developed by Frezza et al. [13] and they were used as positive controls.

2.2. Isolation of genomic DNA
Total genomic DNA was extracted by following procedure of gsync™ DNA extraction kit for animal tissue (Geneaid Biotech Ltd., Taiwan). Then, 1% agarose gel electrophoresis was performed using Mupid at 100 Volt for 30 minutes. To check the quality of DNA genome, the agarose gel was placed into Glite UV Intuitive Gel Documentation System (Pacific Image Electronics Co., Ltd., Taiwan). Brighter DNA band indicated higher DNA concentration (data not shown). Finally, isolated genomic DNA was stored in the freezer at -20°C until used [14].

| Table 1. Oligonucleotide primers of 12S rRNA gene used in this study |
|-----------------|-----------------|-----------------|
| Species        | Nucleotide (5’ to 3’) | PCR Product size |
| Bovine         | Forward: ACCCGGTCATACGATT | 155 bp |
|                 | Reverse: AGTGCCTCGGCTATTTGAGG | |
| Porcine        | Forward: ACCCGGTCATACGATT | 357 bp |
|                 | Reverse: GAATTGGCAAGGGTTGGTAA | |
| Chicken        | Forward: ACCCGGTCATACGATT | 611 bp |
|                 | Reverse: CGGTATGTACGTGCCTCAGA | |

Cahyadi et al. (2018)
2.3. Multiplex-PCR

Multiplex-PCR has been carried out in a total volume of 25 μL containing 12.5 μL Green Master Mix (GoTaq®, USA), 1 μL DNA template, 1 μL of each 10 μM forward primer and reverse primers (Table 1), and 7.5 μL ddH2O (GeneAmp® PCR System 9700, Singapore). The reaction was initiated by pre-denaturation at 95°C for 3 minutes, and then it was followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 30 seconds. It was terminated by final extension at 72°C for 3 minutes [12,15]. To evaluate the PCR product, 2% agarose gels electrophoresis stained with 1st BASE FloroSafe DNA Stain (Axil Scientific Pte., Ltd., Singapore) was conducted at 100 volt for 30 minutes and then it was placed into Glite UV Intuitive Gel Documentation System (Pacific Image Electronics Co., Ltd., Taiwan) to capture photograph. A Benchtop 100 bp DNA ladder (Promega, USA) was used standard size of DNA bands.

3. Results and discussion

The result of PCR product visualization is presented in Figure 1. Positive controls for bovine (S), porcine (B), and chicken (A) were perfectly amplified which are indicated by 155, 357, and 611 bp, respectively. Positive control and replicate samples were used to avoid ambiguity or false-positive results due to an error of PCR procedure [16]. Meat bone meal samples have been tested through PCR twice in this study and they were indicated containing bovine DNA. The PCR products originated from MBM samples consistently showed the existence of bovine genome marked by 155 bp DNA bands in the photograph (Figure 1). Moreover, this study indicated that multiplex-PCR with mt-DNA 12S rRNA as a target region was able to amplify genomic DNA template extracted from rendered products such as MBM. The use of 12S rRNA gene in PCR was previously reported to be able to amplify target regions from treated samples at 160°C with pressure at 300 kPa and it also could detect the existence of species of interest until only 0.01% in the sample [16-18].

Figure 1. Multiplex-PCR products in this study. M is 100 bp DNA marker; S, B, A are bovine, pig and chicken; M1, M2, M3, M1A, M2A, M3A are MBM samples and their replicates.

According to regulation of European Council (EC) No. 1069/2009 about animal by-products regulation, an species is prohibited to be fed by feed or feedstuffs containing itself, therefore porcine is prohibited to be fed by pork and its derivatives and poultry is not allowed to eat feed containing poultry by-products which is mainly arose during slaughtering process [5]. This regulation is also valid for ruminants, feeding of bovine protein to cattle is not allowed. The objective of this act is to ensure feed and food safeties. Moreover, prohibition cannibalism through animal feeding is installed for ethical and authenticity reasons [19]. The existence of bovine DNA in MBMs marketed in Indonesia indicated that samples used in this study was authentic made from cattle and its derivatives.
This finding should be taken into account that these MBMs could only be used for non-ruminant animals. Cattle and other ruminants are banned to be given feed containing those MBMs.

Since 1996, MBM is considered most responsible in spread of BSE, so it is banned to be one of feedstuffs for ruminant’s feed by WHO, OIE, and FAO [6]. In Indonesia, prohibition of MBM as ruminant feed components is started in 2002 in accordance to Ministry of Agriculture Act No. 471/Kpts/OT.210/5/2002 [20]. Meat bone meals processed from rendered ruminant’s by products are still permitted to be an ingredient of poultry and porcine feeds due to both pig and poultry are not susceptible to infection of BSE agents [7,21]. Therefore, three MBM samples evaluated in this study could be used as protein source for non-ruminant animals since only bovine DNA identified in the samples which is indicated by 155 bp DNA band.

4. Conclusion
Meat bone meal samples used in this study were indicated containing bovine DNA and they were allowed and safe to be a component of feed for poultry, porcine, and other non-ruminants. Multiplex-PCR using mt-DNA 12S rRNA was effective, easy, quick and accurate technique to identify species contained in MBM.

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