Polymerase chain reaction to confirm biochemically characterization method of Pasteurella multocida isolate from fatal cases of Septicaemia epizootica in Nusa Tenggara Timur

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Abstract. Septicaemia epizootica (SE) is a common fatal systemic disease in cattle and buffalo due to Pasteurella multocida serotype B:2 in South and Southeast Asia countries, including Indonesia. This infectious agent is generally considered an opportunistic pathogen and located in the nasopharynx of the animal. To support the disease’s diagnosis, an acceptable identification procedure must be established. This study was to confirm bovine P. multocida isolate that has been identified through biochemical approaches at the first step, with the Polymerase Chain Reaction method. The isolate was obtained from the fatal outbreak of cattle in Kupang in 2016 and subjected to be identified using biochemical characterization, but it was time consumed. The API 20NE was applied to identify the isolate and help to save time. The PCR result showed positive for 16 sRNA and kmt genes, both were classified as specific genes, and the capsular serotype was detected in less than 24 hours. It indicates that PCR confirms biochemical technique, and it is an appropriate and faster method in detecting pathogen agents than biochemical one.

Keywords: systemic disease, pathogenic agents, pneumonic pasteurellosis.

1. Introduction

Pasteurella multocida is a pathogen bacterium that normally lives upper respiratory tract of the host. These pathogenic bacteria are the causative agent of Haemorrhagic septicemia in cattle and impact to decrease income of farmers. Hemorrhagic septicemia is also known as pneumonic pasteurellosis, shipping fever, and is popular as ‘gorok’ disease or septicaemia epizootica in Indonesia.

P. multocida isolates are categorized primarily into five groups (A: hyaluronic acid, B, D: heparin, E, and F: chondroitin) based on their capsule antigens and based on lipopolysaccharide (LPS) antigens is classified into 16 serotypes [2-4]. Each serogroup above indicates host and disease-generating specificity and susceptibility. For example, P. multocida serotype A is the causative agent of chicken cholera in poultry, even it also has been isolated from porcine with pneumonia disease in Korea [5,6]. P. multocida B and E are the causative promoter of hemorrhagic septicaemia in cattle and buffalo, and the disease is mainly appropriated in Asia and Africa [7,1]. P. multocida D and F are the causative agent of continuous atrophic rhinitis (PAR) and pneumonic disease in pigs, respectively [8]. Some instances
revealed that they, together with other inhaling pathogen agents, were playing an important part in the porcine respiratory disease complex (PRCD) [9].

Identification of some bacteria isolate can be conducted using the biochemical technique, but it cannot be used to classify them based on their capsular types, biochemical technique is also time-consuming. Current methods are being developed based on genomic DNA analysis. To detect \textit{P. multocida}, molecular approaches targeting particular genes of \textit{P. multocida} bacteria were used to amplify 16S rRNA [10] and \textit{kmt1} genes [11]. PCR technique will amplify part of the specific gene sequence of the bacterium. The comparison of gene sequences can be used to categorize organisms [12].

This study aims to confirm bovine \textit{P. multocida} isolate that was previously identified through biochemical approaches, using the Polymerase Chain Reaction method to get their advanced characteristic.

2. Materials and Methods

These experiments were performed in the bacteriology laboratory at Indonesian Research Centre for Veterinary Science.

2.1 Culture

We used some respiratory and other organs as samples, such as lungs, tonsils, and bones. Firstly, the organ or bone samples were cleaned with alcohol axenic by flaming and cut open with a sterile knife or saw. A few sample quantities were scooped out and chopped. A single drop of this substantial (blood or bone marrow) was placed on the edge of a Blood Agar culture plate (Oxoid) and spread with a flame-sterilized platinum loop, chilly by placed on the medium. At the same time, from the drop material or chopped organ, we cultured them into sterile Brain Heart Infusion (BHI) broth (Oxoid) media. After being incubated overnight, it directly poured and streaked on Casein Sucrose Yeast (CSY) medium.

2.2 Biochemical identification

After incubating at 37°C for around 18-24 hours, one individual colony on the final streak of Blood Agar was detected using the following techniques. They were Gram stain, subcultured on Mac Conkey agar plate, Triple Sugar Iron Agar Test, Catalase Test, Oxidase Test, and completed by API 20 NE kit (BioMérieux, France) according to the product protocols.

2.3. Molecular identification

\textit{P. multocida} isolates were injected into BHI broth (Oxoid) and cultured for 18-24 hours. Genomic DNA was extracted using the QiAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Species of the isolate were determined by PCR using 16S rRNA primers, specific species using \textit{kmt} gene primers, and capsular types using capsule-unique primers shown in Table 1.

3. Results and Discussion

\textit{Pasteurella multocida} mature on basic standard media such as nutrient agar base or enriched media such as casein-sucrose-yeast agar (CSY agar) or tryptose agar with or without 5% sterile blood. Blood of a young calf - the natural host - free of antibodies is better. Here, we use agar base media with 5% sterile sheep blood, and for BHI broth was supplemented with 5-10% Foetal Bovine Serum (Sigma Aldrich, USA). Enhancement of the media and the addition of blood will sell an increase for bacterium [1].

Sub-culturing bacterial isolate was carried out on blood agar essential plates. Bacterial colonies have some characteristics, i.e., smooth, mucoid, convex, colonies with or without causing hemolysis on blood agar, a Gram-negative, rod, and coccobacilli. Isolate failed to grow on McConkey. Then each colony was characterized and continued to recognize using a series of essential and auxiliary biochemical tests following standard procedure.
Table 1. Primers used for the recognition of species, specific species, and capsular types genes in *Pasteurella multocida* isolate.

| Gene function | Target gene | Description | Sequence (5’ – 3’) | Size (bp) | Reference |
|---------------|-------------|-------------|--------------------|-----------|-----------|
| Species identity | 16S rRNA | Identification of family bacteria | AGGCCCTCGGCGTGAAGT | 642 | [13] |
| Specific | kmt1 | Recognition of all *P. multocida* isolates | ATCCGCTATTTAACCAGTGG | 460 | [11] |
| Capsular serotypes | bcbD | Group B cap gene | CATTTATCCAAGCTCCACC | 760 | [2] |

*P. mutocida* can be distinguished from another Gram-negative enteric bacterium by inoculating the culture onto TSI agar. Inoculation culture onto TSI agar helps to distinguish *P. multocida* from another gram-negative enteric bacterium commonly found from intestinal samples. *P. multocida* showed a gradual acid reaction with no gas and no hydrogen sulfide. TSIA’s color was altered from clear–red to clear–orange or soft yellow–red in this experiment. It indicates a slow acid reaction, both in slant and boot TSIA medium without any crack and black spot. The culture showed oxidase and catalase-positive, failed to both produce urease and grow on the McConkey agar medium.

Advance and simple biochemical characterization of *P. multocida* conducted using API 20 NE diagnostic kit following manufacturer’s protocol. A single colony of isolate found on the agar plate was picked up using a sterile loop and diluted into 10 ml of sterile aquadest in a McCartney bottle. The solution was filled into an API strip and incubated at 37°C for 24 hours (Figure 1) using a Pasteur pipet. The strip read to get the score and analyzed online at apiweb.biomerieux.com. The biochemical test of local isolate conducted using API 20NE kit showed it was 96% *P. multocida* (Figure 2).

Biochemical identification using conventional process needs about 18–24 hours for each following process: culture sample into BHI broth medium, inoculation onto blood agar medium, McConkey agar test, and TSIA test. Identification using API 20 NE kit helps to reduce time-consuming about 18-24 hours since culture isolates onto McConkey agar plate can be removed.

Molecular identification of the isolate was being started from DNA extraction, measurement DNA concentration using Nano-drop spectrophotometer, and continued to PCR reaction process. Results of PCR analysis of species identity, species-specific and capsular serotype genes showed that the local isolate can be detected as having all three targeted genes less than 24 hours.

Species identity of the isolate was determined in 642 bp size (Figure 3). 16S rRNA sequencing has represented an essential step for microscopic organisms recognizable proof and fundamental data for their classification [14]. 16S rRNA gene detection is widely identified as the ‘gold standard for bacterial identification due to these specific features [10].

Typing of the strain was attended with the use of PCR analysis applying respectively species-specific and capsular-specific primers for determining specific kmt gene in size of 460 bp (Figure 4) and capsular group B of 760 bp (Figure 5).

![Figure 1. Biochemical identification of *P. multocida* using API 20 NE strip.](image)
Figure 2. Result of Biochemical Analysis using API 20 NE kit to identify *P. multocida* isolate.

Figure 3. Detection of 16SrRNA gene of *P. multocida* isolate.
1: NCTC11668, 2: ATCC12945, 3: Pma, 4: PMb, 5: PMc, 6: PMd (Lampung), 7: NTT

Figure 4. Detection of *kml* gene (specific species) of *P. multocida* isolate.

Figure 5. Detection of capsular serotype B (*bchD* gene) of *P. multocida* isolate.
4. Conclusion
Polymerase Chain Reaction was successfully performed to confirm biochemical identification which was carried out conventionally or using kits. In addition to saving time, this technique can identify more specifically the capsular gene level of the *P. multocida* isolate.

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