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RESEARCH ARTICLE

SEROTYPING OF PASTEURELLA MULTOCIDA BASED ON CAPSULES IN DUCKS IN KELANTAN, MALAYSIA

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ABSTRACT

The present study was carried out on 65 duck liver to investigate the serotypes of *Pasteurella multocida* causing fowl cholera in the state of Kelantan, Malaysia. These samples were obtained from different farms in Kelantan which was preparing for marketing purpose during the period from August to December 2013. Bacterial isolation, morphological identification, biochmical properties, and bacterial DNA extration for PCR revealed that out of 65 samples 4 were *P. multocida* positive, confirmed by PCR. The 4 samples identified produced species-specific 460bp band with KMT1T7 and KMT1SP6 primers.PCR serotyping using CAPA-FWD and CAPA-REV primers revealed that among 4 *P. multocida* positive samples, 1 was type A strain having 1044 bp, and the other 3 strains were untypeable. The present study revealed that *P. multocida* type A strain is prevalent in Kelantan.

INTRODUCTION

The major constraints of duck farming in Malaysia is the outbreak of Pasteurellosis caused by *P. multocida*. Environmental contamination from disease birds is a primary source for infection. In Malaysia in 2004, fowl cholera caused 50% of the mortalities in 400 populations of Muscovy duck in the district of Kelantan (2005, Annual report of Regional Veterinary Laboratory, Kota Bharu-unpublished report). *P. multocida* infects not only ducks, but also mammals causing diseases such as haemorrhagic septicaemia and shipping fever in cattle (Carter and De Alwis, 1989), fowl cholera in poultry (Glisson *et al*., 2003), and pneumonia in pigs (Chanter and Rutter, 1989). There are a few serogroups of *P. multocida*) with five distinct capsular serogroups which are A, B, D, E and F (Rimler and Rhoades, 1989). Fowl cholera in the ducks is caused by serogroups A and D (Rhoades and Rimler, 1989) whereas pneumonia and atrophic rhinitis in pigs are caused by serogroups A and D (Chanter and Rutter, 1989). In cattle, serogroup A caused pneumonia (Frank 1989), however serogroups B and E are associated with hemorrhagic septicemia (Carter and Alwis, 1989). In ducks, Pasteurellosis is characterized by fever, depression, anorexia, mucous discharge from the mouth and nostrils, diarrhea and increased respiratory rate. The death in ducks is sporadic and postmortem findings shows enlarged liver and septicaemia. Accurate laboratory detection of *P. multocida* depends on the isolation and identification of suspect bacterial colonies by microscopy and biochemical tests. Samples taken immediately from animals that died of suspected pasteurellosis yield almost pure cultures of *P. multocida* from the heart blood, liver, spleen, bone marrow and lung. However, isolation of *P. multocida* can prove difficult during field surveys of carrier ducks when samples are taken from the nose or throat with mixed infection where subculturing is then required to obtain a pure culture isolation of the causative organism. Preparation of antisera for serotyping procedures is time consuming which results in definitive serological determination is impractical for most laboratories in countries where HS is endemic (Rimler and Roades, 1989).

This may lead to an increased lag between the collection of samples and serotype identification when lengthy transportation is required for the samples to reach a laboratory prior to definitive serotyping procedures. In recent years, genotypic methods of bacterial identification have proved beneficial in overcoming some limitations of traditional phenotypic procedures. Nucleic acid-based assays allow the detection of organisms directly from clinical samples or from small amounts of cultured bacterial cells, thus dramatically improving the sensitivity and decreasing the time required for bacterial identification. PCR has been particularly useful in this regard, with the use of primer sequences designed to

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facilitate identification at any level of specificity: strain, species, genus, or all members of a domain (Relman and Persing, 1996). Therefore, in the present study, we diagnosed the *P. multocida* from farms of Kelantan based on cultural characteristics, bacterial identification and characterization, and by capsular typing following polymerase chain reaction (PCR).

MATERIALS AND METHODS

Sample Collection

A total of 65 samples of duck liver (prepared for marketing purpose) were collected from different sites which are local farm in Melor district, Kelantan from August to December 2013. Each samples were dip into 70% ethanol and cut transversely to open the inner sides of the liver. The inner sides then chopped onto Columbia Horse Blood Agar plate and MacConkey Agar Plate before incubating at 37°C overnight for primary culture of bacteria. Each colony on Columbia Horse Blood Agar then forwarded for secondary culture to collect pure colony of the bacteria.

Gram Staining

The suspected colonies then were prepared for gram staining to check the morphology of the bacteria and to compare with positive control gram staining of *P. multocida*.

Biochemical Test

Gram negative bacteria were tested with biochemical tests which are Citrate test, Catalase test, Triple Sugar Iron test, Simmon Citrate test, SIM test, MRVP test, Urease test and Indole test. After incubation for 24 hours at 37°C, results for the biochemical tests were interpreted. The samples with positive growth of *P. multocida* were subjected to repeat the test to avoid false positive result. This step is important in confirmation of *P. multocida* identification and characterization.

DNA extraction

The sample from biochemical tests suspected for *P. multocida* was cultured in Brain Heart Infusion (BHI) broth for 24 hours to grow the bacteria. The bacterial cell from BHI broth were harvested for DNA extraction. The procedure for DNA extraction was done using DNA Extraction Kit Vivantis according to the protocol provided by the company. Purity and quality of extracted DNA was checked using nanophotometer UV absorption at 260/280nm. 1.0% Agarose Gel was prepared to run gel electrophoresis at 85V, 400mA for 45 minutes and view the band under UV light.

Capsular typing by Polymerase Chain Reaction (PCR)

Capsular genes detection by PCR for all serogroups was done according to the method described by Townsend *et al*. (2011) with some modifications. A singleplex-PCR was used to confirm the strains of *Pasteurella multocida* and to determine if the strains were of serogroups A,B,D, E and F. Each PCR reaction contained 50ng of DNA template, 1X PCR Buffer, 2.5mM MgCl2 buffer, 0.25mM dNTPs, 0.3µM forward and 0.3µM reverse primers and 1.75U Taq Polymerase. PCR conditions consisted of an initial denaturation at 95^0 C for 10 min followed by 35 cycles of denaturation (95^0 C, 30s), annealing (55.1^0C, 30s), extension (72^0C, 30s), and a final extension of 72^0C for 7 min. The primer concentrations used were: Primers KMT1T7, KMT1SP6 (*P.multocida*), A-FWD, A-REV (serogroups A), B-FWD, B-REV (serogroups B), D-FWD, D-REV (serogroups D), E-FWD, E-REV (serogroups E), F-FWD and F-REV (serogroups F). As a positive control, equal amount of 50 ng DNA template from reference strain for serogroup A were mixed and 1 ml of the mixture was used for every PCR run. As negative control, one PCR reaction were added excluding DNA template for every run.

RESULTS AND DISCUSSION

The primary colonies were roughly rounded in the columbia horse blood agar. Each colony on columbia horse blood agar forwarded for secondary culture of the bacteria and the secondary culture media revealed medium size (3 mm in 24 hours), whitish to creamy colour and mucoid colonies (Fig. 1) which were suspected to be *P. multocida*. After gram staining, the smear revealed presence of Gram-negative bipolar cocco-bacillus (Fig. 2).Biochemical analysis revealed indole and sulphure positive.

Fig.1. Colony morphology of *P. multocida* on blood agar medium. The colony are whitish in colour, medium sized, mucoid with unpleasant smell.

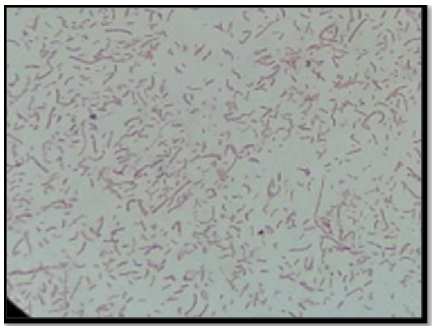

Fig. 2. Gram staining of suspected colony of *P. multocida* show gram negative cocco bacillary bacteria.

The bacteria produced bubble in catalase examination and blue colour on strip revealed oxidase positive. Using vivantis DNA extraction kit, and PCR technology, 4 samples revealed species-specific 460bp band with KMT1T7 and KMT1SP6 primers. PCR serotyping using CAPA-FWD and CAPA-REV primers showed that among 4 *P. multocida* positive samples, one was type A strain having 1044 bp. The morphology, colony characteristics and biochemical findings of the present study in accordance with the result of the previous authors (Baki *et al*., 1993; Ganti and Shashtri, 2003; Arumugam *et al*.,

2011). Systemic investigation on the occurrence of *P. multocida* has previously been carried out on village chickens (Curtis and Ollerhead, 1981; Muhairwa *et al*., 2001) but not on traded (slaughter and live market) family chickens and ducks. In the present study out of 65 samples, only four liver were positive for Pasteurellosis (6.15% infection rate). This is due to the fact that, the liver were processed in the farms in hygienic condition. The isolation rate of *Pasteurellosis* is high among bird sampled at markets level or transportation from farm to market, due to handling (Mbuthia *et al*., 2008).

The important feature of the present study was the number of untypeable strains (3 out of 4 – 75%) which could not be assigned to any capsular serogroup. A number of studies have reported the presence of untypeable strains using the modified PCR method (Townsend *et al*., 2001). These previous studies have reported levels between 2% to 9% of isolates as untypeable (Davies *et al*., 2003a, 2003b; Jamaludin *et al,* 2005; Ewers *et al*., 2006). In the present study the untypeable strains detected are significantly higher. There is limited information on the serogroups of *P. multocida* in Kelantan state of Malaysia. This study has confirmed the presence of *P. multocida* serotype A in the farms of Kelantan. However, Arumugam *et al*., (2011) reported serotype A, and D in the duck of Malaysia. This may be due to the fact that serotype A and D is not equally distributed in all the state of the Malaysia. Further studies on larger numbers of strains from the duck in this state needs to be undertaken as well as the untypeable strains will be characterized molecularly by surface protein analysis of the bacteria, western blotting, 2-D gel electrophoresis and by animal experimentation in our laboratory.

Conclusions

Molecular analysis of the present study revealed that the *P. multocida* serotypes A and B are prevalent in the state of Kelantan, Malaysia and that of untypeable strains need to be further study

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