OUTBREAK OF PASTEURELLOSIS IN CAPTIVE EMU BIRDS AND DETECTION OF VIRULENCE GENES IN P. MULTOCIDA ISOLATES

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ABSTRACT

Avian pasteurellosis caused by Pasteurella multocida was reported among 180 species of wild birds but not reported so far in emu birds. Several emu birds were reported to have died over a period of one week in an organized emu farm. Necropsy findings revealed typical haemorrhagic lesions and characteristic bipolar organism, suggestive of avian pasteurellosis. Two Pasteurella multocida isolates from heart blood were obtained and characterized. Pasteurella multocida Polymerase Chain Reaction (PM-PCR) using the specific primers confirmed these isolates as P.multocida. Capsular PCR revealed the presence of serogroup 'A'. Among four important virulent genes in P.multocida two genes, pfhA and hgbB, showed significant association.

Key Words: Avian pasteurellosis, emu, Pasteurella multocida, PM-PCR

INTRODUCTION

Avian pasteurellosis is a septicaemic disease of wild and domestic birds. The causative organism by Pasteurella multocida, is thought to be transmitted either by inhalation of a bacteria-laden aerosol or by consumption of contaminated water or food (Botzler, 1991). It causes respiratory disease in more than 180 species of wild birds (Samuel et al 2005.). Emu (Dromaius novaehollandiae) are flightless prehistoric birds that originated about 80 million years ago in Australia and belong to members of the ratite family along with ostrich, rhea, cassowary and kiwi. Pasteurella spp. are important primary and opportunistic pathogens as well as common commensals of the upper respiratory tract of various domestic and wild animals. The evidence for carrier birds among wild fowl is inconsistent and similarly, presence of virulent and non-virulent carriers in domestic poultry has been known for many years (Wobeser 1992). But so far P. multocida infection is not reported in emu birds.

In P. multocida there are four important virulence genes, two were related to iron metabolism...
and two were independent non-sidophore mediated acquisition of iron mechanism have been identified. (Ewers et al., 2006). Virulence genes pfhA, hgbB, thpA and toxA have an important role in epidemiological studies.

**MATERIALS AND METHODS**

**Isolation**

Emu birds reared in a private farm in Tamil Nadu, showed respiratory disorder and diarrhoea. Mortality of 3-5 birds per day for one week was recorded. Five emu birds was subjected to necropsy examination. Heart blood swabs collected from emus suspected to have died of avian pasteurellosis were inoculated in brain heart infusion broth and incubated at 37°C overnight.

Two hundred microlitre of the above overnight broth cultures were inoculated subcutaneously in swiss albino mice. Heart blood samples aspirated from the dead mice were streaked directly onto blood agar and P. multocida selective agar (PMSA) and incubated at 37°C for 24 hours. The samples were also streaked on MacConkey agar.

The colonies suggestive of P. multocida were subjected to biochemical tests for identification. The biochemical tests included IMVIC, sugar fermentation test and catalase and oxidase test. Impression smears from heart, liver and spleen of the dead mice were also stained by Leishman method and observed.

**Antibiotic sensitive assay**

Antibiotic sensitive assay was performed for the P. multocida isolates with 7 antibiotics (Enrofloxacin, Gentamicin, Tetracycline, Streptomycin, Ampicillin, Cephalexin and Ciprofloxacin) as per the disc diffusion method of Kirby-Bauer (Bauer et al., 1966).

**Molecular Characterization and Confirmation**

**DNA Isolation by High Salt Method**

DNA was extracted from the culture by high salt method as described by Senthilkumar and Ramadass (2000). Overnight cultures were centrifuged at 10,000 rpm for 20 min. The pellets were washed with PBS twice. The resulting pellets were suspended in 0.5 ml of solution I (10mM Tris HCl, 10mMKCl, 10mM MgCl2,2mM EDTA) and in 0.5ml of solution II (10mM Tris HCl, 10mMKCl, 10mM MgCl2,2M EDTA,0.4M NaCl) and incubated at 370C for 15 min in water bath. Fifty micro litre of 10% SDS and 250µl of 6 M NaCl were then added and centrifuged at 10,000 rpm for 5 min at 40C and ethanol precipitated. The pellets were resuspended in LTE (Low Tris EDTA) buffer and stored at -200C until used.

**P. multocida species specific PCR (PM-PCR)**

The species specific primers designed by Townsend et al.,(1998) KMITT7 and KMITISP6 were used to amplify the KMT1 gene sequences in P. multocida. The PCR reaction mixture and the thermal cycle protocol were as follows. Initial denaturation - 94°C for 5 min, followed by 30 cycles, each cycle consisting of 3 steps- denaturation at 95°C for 1 min, annealing at 49°C for 30 sec, Extension at 72°C for 80 sec. Final Extension was carried out at 72°C for 5 min.

**Capsular PCR typing**

The P. multocida capsular serogroup specific primers designed by Townsend et al. (2001) were used for capsular PCR typing. The serogroup A specific primers hya D and hya C were used to amplify capsule biosynthetic loci of serogroup “A”. The thermal cycle protocol was as follows. Initial denaturation at 95°C for 5 min, followed by 30 cycles, each cycle consisting of 3 steps- denaturation at 95°C for 30 sec, annealing at 49°C for 30 sec, Extension at 72°C for 80 sec. Final Extension was carried out at 72°C for 5 min.
**Multiplex virulence PCR typing**

The *P. multocida* virulence gene primers for pfhA, hgbB, tbpA and toxA genes designed by Sina Atashpaz et al. (2009) were used for virulence PCR typing. The thermal cycle protocol was as follows., Initial denaturation at 94°C for 5 min, followed by 30 cycles, each cycle consisting of 3 steps denaturation at 94°C for 45 sec, annealing at 54°C for 50 sec, Extension at 72°C for 50 sec. Final Extension was carried at 72°C for 10 min.

**RESULTS**

Five emu birds on necropsy examination revealed congestion of the carcasses, necrotic foci and multiple petechiae on liver and spleen, congestion and edema of lungs and scattered haemorrhage in the mucosa of small intestine.

*P. multocida* isolates showed dew drop, mucoid, non haemolytic colonies in blood agar. No growth was observed in MacConkey agar. Grams staining of the smears revealed characteristic gram negative coccobacillary organisms. *P. multocida* isolates were subjected to mouse bio assay and were found to be virulent with Mean Death Time (MDT) between 12-18 hrs.

Heart blood smear, liver and spleen impression smear from dead mice showed characteristic bipolar organism on Leishman staining. Biochemical tests like indole, Nitrate reduction, Oxidase and Catalase production are positive (Table-1)

Antibiotic sensitivity assay revealed moderate sensitivity to Ciprofloxacin, and resistance to Enrofloxacin, Gentamicin, Tetracycline, Streptomycin, Ampicillin, Cephalexin.

*P. multocida* species specific PCR (PM-PCR) showed 460 bp amplified product (Fig-1) there by confirming the two isolates as *P. multocida*. Capsular PCR typing of *P. multocida* isolates revealed 1044 bp product size (Fig-2) there by confirming the two isolates as capsular Serotype 'A'.

Multiplex Virulence PCR typing showed amplified product of 275 bp and 499 bp indicated the presence of pfhA and hgbB genes (Fig-3).

**DISCUSSION**

The epidemiology of fowl cholera outbreaks is complex (Christensen & Bisgaard, 2000). Avian species have different susceptibility to the disease, but the course of an outbreak depends on other factors as well. When outbreaks involve more than one species, there tends to be sequential mortality and some species suffer disproportionately higher mortality than others. A high population density is a predisposing factor as probably is stressful weather, such as precipitation and sudden change from hot to cold weather (Botzler, 1991).

Necropsy examination revealed severe gross lesions similar to the observation in Ring Necked Pheas as reported by Einum et al 2003.

The isolates showed typical cultural characteristics of *P. multocida* and similar findings were reported in fowl cholera in chicken (Prabhakar (1995) and OIE (2004). The Mean Death Time (MDT) of the two isolates in mice were same as that of the finding of Diallo et al (1995) and Suresh babu (2003) with regard to Mean Death Time in chicken.

Biochemical tests results coincided with the findings of Kawamota et al. (1990) and OIE (2004). Antibiotic sensitivity assay findings are in accordance with Dwight et al (1989) who reported antibiotic resistance in avian isolates.

Pasteurella multocida species specific PCR (PM-PCR) assay developed by Townsend et.al (1998) was used for this study to identify the subspecies of *P. multocida* by amplifying 460 bp DNA fragment within KMT gene using the Primers KMTISP6 and KMTIT7. Based on standard
molecular weight marker (100 bp Marker), the molecular weight of the PCR products of the isolates was found to be 460 bp, as reported by Townsend et al (1998).

Fowl cholera in avian species is caused by various capsular and somatic serotypes Pasteurella multocida (Kumar et al 1996) Capsular serotyping revealed that the isolates belonged to capsular 'A' type. Pasteurella multocida from waterfowl and associated avian species possessed the capsular type ‘A’ as per the findings of Zinkl et al, (1977) and Price & Brand (1984).

Chengmin Wang et al. (2009) reported fowl cholera in wild waterfowl in China and causative agent Pasteurella multocida capsular type ‘A’, when inoculated in Muscovy ducks caused disease. Fowl cholera was reported in wild geese in captivity caused by P multocida serotype 'A' (Purushothaman et.al.,2008). Therefore it is ascertained that P. multocida serotype ‘A’ is found to be predominant among waterfowls and associated avian species. The origin of P. multocida, as related to this outbreak, is unclear, but it may have originated due to undue stress of unseasonal rainfall that occurred during the period of outbreak or from domestic birds as reported by Botzler (1991).

In our study 100% prevalence of pfhA and hgbB gene was noticed in P.multocida isolates of avian origin whereas tbPA and toxA gene was not present in any of the isolates. High prevalence hgbB gene was noticed among P.multocida isolates of avian origin and this coincides with the findings of Ewers et al. (2006). High prevalence pfhA gene was noticed in this current study but Ewers et al. (2006) reported 45% percentage of pfhA gene among P.multocida isolates of avian origin, tbPA gene was not present in any of the P.multocida isolates of avian origin and toxA gene was present in only less than 5% of the avian isolates and this coincides with the findings of our study.

In summary, the occurrence of avian pasteurellosis in emu is reported for the first time and the presence of the virulence genes detected in the isolates. Among four virulent genes considered as epidemiological markers only two genes, pfhA and hgbB, found in the isolates causing the disease in Emu. More number of samples from different avian species should be studied to determine the genes responsible for the disease and for use as epidemiological markers.

ACKNOWLEDGEMENT

The authors acknowledge Indian Council of Agricultural Research (ICAR) and Dean, Madras Veterinary College for providing facilities to carry out the research work.

REFERENCE


Sina Atashpaz A.B., Jalal Shayegh, C and Mohammad Saied Hejazi, D., 2009 Rapid virulence typing of Pasteurella multocida by multiplex PCR. Research in veterinary science., 87: 355-357


Outbreak of ....

Fig - 1

P. multocida species specific PCR (PM-PCR)

Fig - 2

P. multocida Capsular PCR
**Fig: 3**

**Multiplex Virulence PCR typing**

![Multiplex Virulence PCR typing](image)

E1 - *P. multocida* of emu origin  
E2 - *P. multocida* of emu origin  
M - 100bp Molecular weight marker

**Table -1**

**Biochemical characteristics of *P. multocida* isolates**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Indole</th>
<th>MR test</th>
<th>VP test</th>
<th>Simmons Citrate</th>
<th>H₂S Production</th>
<th>Nitrate Reduction</th>
<th>Lysine decarboxylase</th>
<th>Ornithine decarboxylase</th>
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<td>-</td>
<td>-</td>
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<td>+</td>
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<td>-</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<th>Samples</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Urease</th>
<th>ONPG</th>
<th>Phenylalanine</th>
<th>Esculin hydrolysis</th>
<th>Arginine</th>
<th>Dulcitol</th>
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