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Biotyping and antibiogram of *Riemerella anatipestifer* from ducks in Kerala

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ABSTRACT

New duck disease caused by *Riemerella anatipestifer* is a new disease emerged in Kerala from 2008 onwards. Six *R. anatipestifer* isolates responsible for the disease were isolated from suspected ducks from different outbreak areas of the state and were identified. Since the ecological, morphological and cultural characteristics of *R. anatipestifer* are more or less similar to *Pasteurella multocida*, the disease is often confused with duck pasteurellosis and misdiagnosed. *R. anatipestifer* infection is also characterized by the presence of bipolar organisms in blood smear and impression smears of organs as in the case of *P. multocida*, but the size is little larger. The detection and identification of the causative bacterium, from ducks with signs and lesions consistent with the acute or chronic form of the disease, is one of the most important aspects of disease diagnosis. Hence, a study was conducted to isolate the agent of new duck disease and stating its differential biotyping characters from that of *P. multocida*. They were differentiated using tests like indole production, gelatin liquefaction, ornithine decarboxylases utilization and fermentation of glucose. The antibiogram pattern was determined to advocate the choice of drug for the purpose of treatment. All the *R. anatipestifer* isolates were sensitive to chloramphenicol, ciprofloxacin, enrofloxacin, norfloxacin, gentamicin, clindamycin, doxycycline and cefuroxime.

KEY WORDS: ANTIBIOGRAM, BIOTYPING, KERALA, NEW DUCK DISEASE, *PASTEURELLA MULTOCIDA, RIEMERELLA ANATIPESTIFER*

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INTRODUCTION

God's gift of beautiful water bodies at various localities of Kerala are acting as ideal environment for duck rearing. Regular vaccination against duck plague and duck pasteurellosis carried out in the state greatly reduced their incidence. When we succeed in controlling the existing disease, due to known and unknown global environmental changes, several new diseases are emerging. One such disease is the new duck disease in Kerala, reported since 2008 (Priya *et al*., 2008). It is an enzootic, contagious, often primary septicemic disease of domesticated ducklings (Fulton and Rimler, 2010).

In addition to ducks, it also infects geese, turkey, chicken, wild birds and domestic pigs (Segers *et al*., 1993). In young ducklings, it results in a mortality rate as high as 75 per cent and in adult birds, it ranges from 20 to 40 per cent. The causative agent is *Riemerella anatipestifer*, a Gram- negative rod shaped, non-motile, non-sporulating bacterium.

In India, the disease has been reported in ducks from Assam and Kerala (Shome *et al*., 2004 and Priya *et al.,*2008). Both *R. anatipestifer* and *Pasteurella multocida* reveal bipolarity in blood smear on Lieshman's / Giemsa staining.Both the organisms share common ecological and morphological characters . Hence, the field veterinarians are often unable to distinguish these two organisms due to their phenotypic similarity. Here comes the need of isolation and identification of the agent. The present study discussed in detail on direct microscopic examination, right clinical samples to be collected, selection of cultural media and its incubation condition and the differential biochemical characters of *R. anatipestifer* from that of *P. multocida*. These parameters are highly useful at field level to confirm the disease, (Sun *et al*., 2012, Pala and Radhakrishnan 2014., Soman *et al*., 2014).

MATERIAL AND METHODS

Live and dead ducks (128) from the disease suspected outbreak areas were brought to the Department of Veterinary Microbiology was used for sample collection. Detailed post mortem examination was conducted to observe various gross lesions. Heart blood smears and impression smears of liver and spleen were stained by Leishman's stain for the presence of bipolar organisms. Samples of heart blood, liver, spleen, lungs and brain were collected aseptically and streaked on ten per cent bovine blood agar. They were incubated microaerophilically in a candle jar at 37°C for 48 hours. The bacterial isolates were identified based on morphological and staining reactions, cultural and biochemical characters. Since *P. multocida* is the most confusing organism with *R. anatipestifer*, duck isolate of *P. multocida* serotype A (maintaining in the department) was used for comparison as a negative control. Antibiotic sensitivity pattern of the isolates was determined by standard disc diffusion method (Bauer *et al*., 1966).

RESULTS AND DISCUSSION

Examination of heart blood smears and liver impression smears revealed bipolar organisms which are relatively larger in size than *P. multocida*, indicating the importance of examination of heart blood and impression smears from liver and spleen. Pillai *et al*. (1993) also noted the size difference of bipolarity between these two organisms.

Since the clinical signs and gross lesions of new duck disease are similar to diseases like duck pasteurellosis and *E. coli* infection, the gold standard method of diagnosis is the isolation of the bacteria from clinical materials in suitable media. So the isolation was tried from heart blood, lung, liver, spleen, ovary and brain. In acute stage of the disease, the organism could be readily isolated from heart blood, liver, spleen, lungs and brain (Pathanasophon *et al.,* 1994). Bisgaard (1995) suggested that *R. anatipestifer* often resulted in chronic salpingitis in surviving duck and geese. So isolation was also tried from ovary of the infected bird. According to Gooderham (1996), the best source of isolation of the organism was brain.

In this study, though isolates were obtained mainly from heart blood and liver, chance of contamination was less in brain. The primary isolation was carried out in five to ten per cent bovine blood agar. According to Rimler *et al*. (1998) no selective and/or indicative media had been used for the isolation of *R. anatipestifer* and the isolation of the organism from clinical materials was sometimes difficult due to the overgrowth of other organisms (Higgins *et al.,* 2000). Chocolate agar (Leavitt and Ayroud, 1997), ovine blood agar (Crasta *et al.,* 2002) and ten per cent bovine blood agar (Priya *et al*. 2008 and Pala *et al*. 2014) have reported to be useful for the primary isolation of *R. anatipestifer*.

The incubation carried out in a candle jar with mild CO_2 tension at 37°C for 48 h was found to be optimum for the culture of *R. anatipestifer* from clinical materials. Smith *et al*. (1987) suggested that the organism preferred microaerophilic environment for initial isolation. Segers *et al*. (1993) reported that the organism grew best at temperature 35 to 37°C after a primary isolation in a CO_2 enriched atmosphere. The findings of the present study are in agreement with the observations made by earlier workers.

Following incubation of clinical samples in bovine blood agar, convex, entire, transparent and butyrous colonies suggestive of *R. anatipestifer* obtained from six birds were designated as RA1 to RA6. These observa-

FIGURE 1. Haemolysis produced by RA2 on blood agar

tions are in accordance with the findings of Smith *et al.* (1987) and Songer and Post (2005). All the isolates were non-haemolytic on blood agar, except one (RA2), which produced a clear zone of haemolysis after 48 h of incubation (Fig. 1), indicating it may be a different strain or serotype, since more than 20 serotypes of *R. anatipestifer* have been reported worldwide (Sandhu. 2008). Hinz *et al.* (1998b) recorded that among 123 field strains of *R. anatipestifer,* 25 strains displayed β haemolysis on blood agar after 24 h to 48 h of incubation. In the present study, out of the 128 birds screened, samples from six birds were showing colonies suggestive of *R. anatipestifer.*

Smears from culture, stained by Gram's staining revealed Gram negative organism with a variable morphology varying from short rods to filamentous forms (Fig. 2). Similar findings were reported by Baba *et al.* (1987) and Leavitt and Ayroud, (1997). The biochemical characteristics of the isolates and its comparison with DP1 are given in Table 1. The isolates did not grow on MacConkey agar. They were catalase and oxidase positive and unreactive to O-F test, as reported by Carter and Wise (2004) and Pala *et al.* (2013). According to Hinz *et al.* (1998a) and Ryll *et al.* (2001), *R. anatipestifer* is characterized more by the absence than the presence of specific phenotypic properties. The second stage biochemical reactions used for characterization of *R. anatipestifer* (Segers *et al.,* 1993) were almost identical for all the six isolates. Variations were observed only in the presence of urease and fermentation of sugars. Similar findings have been reported by Pillai *et al.* (1993), Vancanneyt *et al.* (1999), Bernardet *et al.* (2002) and Shome *et al.* (2004). According to OIE (2008), *P. multocida* and *R. anatipestifer* could be differentiated using tests like indole production, ornithine decarboxylase utilization and gelatin liquefaction. On the basis of morphological, cultural and biochemical characteristics, all the isolates were identified as *R. anatipestifer* and were differentiated from *P. multocida.*

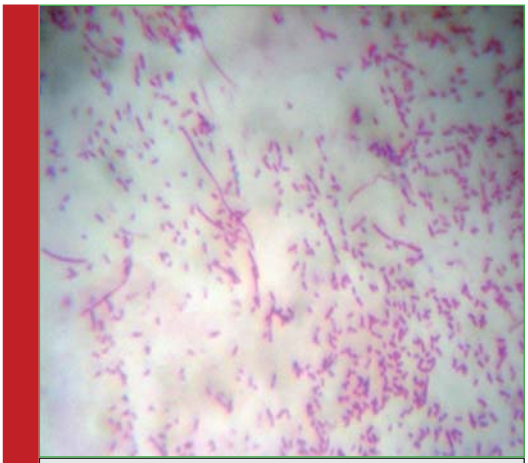

FIGURE 2. Variable morphology of *R. anatipestifer*

Once the organism reaches the brain, chemotherapy is of limited value. Hence, a variety of chemotherapeutic agents have been used in the early stage of the disease itself to treat the infection. As there is often a wide variation in the responsiveness of *R. anatipestifer* to these agents, *in vitro* drug sensitivity testing is essential for the selection of an appropriate drug in a given situation. All the isolates used in the present study were subjected to antibiotic sensitivity testing. Among the 26 antibiotics used, ciprofloxacin, enrofloxacin, norfloxacin, doxycycline, gentamicin, clindamycin, cefuroxime and chloramphenicol appeared to be the most effective drugs as all the isolates tested were found to be sensitive to these agents. Sensitivity to enrofloxacin against *R. anatipestifer* was reported by Turbahn *et al.* (1997). With regard to the sensitivity of ciprofloxacin, gentamicin, chloramphenicol and doxyxycline, the results of the present study are in agreement with those of Shome *et al.* (2004) and Priya *et al.* (2008).

All the isolates showed resistance to methicillin, metronidazole, oxacillin, penicillin G, polymyxin B, erythromycin and sulphadiazine. Other antibiotics tested showed variable sensitivity pattern (Fig. 3). Several workers reported the high sensitivity of *R. anatipestifer* to penicillin G, erythromycin and polymyxin B (Baba *et al.,*1987; Pathanasophon *et al.,* 1991 and Pathanasophon *et al.,* 1994). Chang *et al.*,(2003) conducted *in vitro* and *in vivo* antibiogram using ceftiofur and 16 commonly used antibiotics against 50 isolates of *R. anatipestifer* . Their results revealed that penicillin, cephalothrin, ceftiofur, chloramphenicol, flumequine and kanamycin are the effective antibiotics.

In contrast to that, the study conducted by Zhong *et al.*, (2009) showed that the isolates were resistant to penicillin, ampicillin, tetracycline and sensitive to enrofloxacin, chloramphenicol and neomycin.

Table 1: Biochemical characteristics of *Riemerella anatipestifer*

TESTS	RA1	RA2	RA3	RA4	RA5	RA6	DP1
Gram's reaction	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-
Growth microareobically	-	-	-	-	-	-	-
Growth aerobically	-	-	-	-	-	-	-
Growth on MacConkey agar	-	-	-	-	-	-	-
Haemolysis on blood agar	-	+	-	-	-	-	-
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
O-F test	-	-	-	-	-	-	F
Indole production	-	-	-	-	-	-	+
Methyl-red test	-	-	-	-	-	-	-
Voges-Proskauer test	-	-	-	-	-	-	-
Urease	+	-	-	+	+	+	-
H2S production	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	+
Citrate utilization	-	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+	+	-
Ornithine decarboxylase	-	-	-	-	-	-	+
Sugar fermentation							
Dextrose	-	-	-	+	+	-	+
Galactose	-	-	-	+	-	-	+
Lactose	-	-	-	-	+	-	-
Fructose	-	-	-	-	+	+	+
Sucrose	-	+	-	+	-	+	+
Xylose	-	-	-	-	+	-	+
Mannose	-	-	-	-	-	-	-
Maltose	-	-	+	-	+	+	-
Mannitol	-	-	-	-	-	-	+
Sorbitol	-	-	-	-	-	-	+
Dulcitol	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	+
Trehalose	-	-	-	+	-	+	-
Melibiose	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Raffinose	-	+	-	-	-	-	-

Zhang *et al*. (2014) stated the usefulness of levamizole as immunostimulant in the administration of adjuvanated vaccine. Zhong *et al*. (2009) suggested that *R. anatipestifer* drug resistance profiles changed over time. So to reduce the irresponsible use of antibiotics, disc diffusion analysis should be done for effective antibacterial treatment.

Earlier, Sun *et al*. (2012) reported the prevalence of multi- drug resistant *R. anatipestifer* isolates from China. Manju *et al*., (2014) noticed that ciprofloxacin, enro-

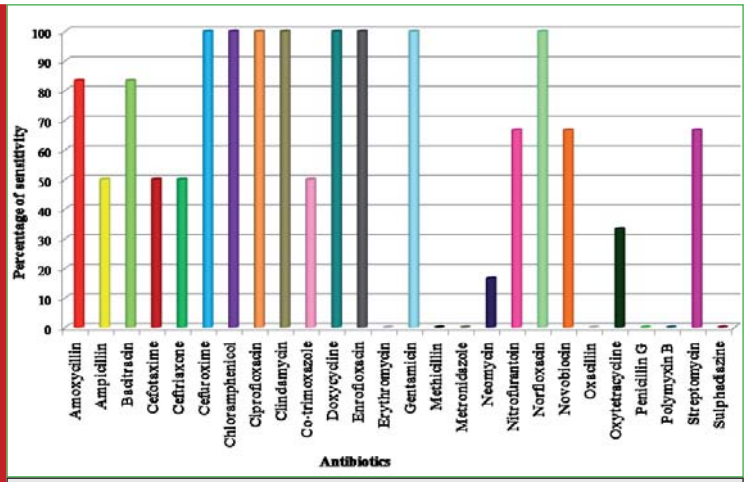

FIGURE 3. Antibiotic sensitivity pattern of *R. anatipestifer* isolates

floxacin and gentamicin gave a wider zone of inhibition where as the *R. anatipestifer* isolates tested were resistant to amoxicillin, chloramphenicol and co-trimoxazole. The variations in the antibiogram of the isolates in the present study could be attributed to the indiscriminate use of antibiotics either to treat the disease condition or their increased use as feed additives, which might have resulted in acquired drug resistance.

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