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Plasmid Profile and Antibigram of Isolates of *Pasteurella multocida* from Ducks in Kerala, India

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Abstract: Twenty five isolates of *Pasteurella multocida* from ducks were assayed for the presence of plasmids in seeking to determine whether any correlation exists between the presence of plasmids, pathogenicity and sensitivity to antimicrobial agents. Twenty two isolates carried plasmids. The plasmids ranged in size from 46.2 to 0.8 kbp. Two plasmids profiles were detected. No correlation was found between the presence of the plasmids and pathogenicity and resistance to antimicrobial agents. The drug of choice for the treatment of duck pasteurellosis in the geographical location is Enrofloxacin, pefloxacin or chloramphenicol.

Key words: *Pasteurella multocida*, plasmids, pathogenicity, antibiogram

INTRODUCTION

Plasmids are covalently closed circular DNA stably inherited in an extrachromosomal state. They harbour the genes responsible for transmissible drug resistance, colicin, haemolysin production, symbiosis and nitrogen fixation by bacteria (Stanisich, 1988).

Several workers have undertaken studies to determine the presence of plasmids in numerous strains of *P. multocida* isolated from several species of animals and birds and to investigate the correlation between antibiotic resistance profiles and the presence of plasmids (Berman and Hirsh, 1978; Hirsh *et al.*, 1981; Haghour *et al.*, 1987; Price *et al.*, 1993; Shivshankara *et al.*, 2000). Very little information is available about the plasmid profiles of *P. multocida* isolated from ducks and so the present investigation was taken up with this objective.

MATERIALS AND METHODS

Pasteurella Strains

Twenty-five isolates of *P. multocida* (DP1 to DP 25) obtained from ducks from different regions of Kerala, India, were used in the study. A reference isolate of *P. multocida* from fowl obtained from the Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar, India was used for comparison.

The bacterial isolates were identified based on morphology, cultural characters and biochemical reactions as described by Barrow and Feltham (1993). The isolates were also confirmed as *P. multocida* by amplification of KMT1 gene (Townsend *et al.*, 1998).

Pathogenicity Testing of Isolates

Swiss albino mice, six to eight weeks of age were used for pathogenicity testing. Each group of mice were inoculated intra-peritoneally with 0.1 mL of inoculum containing 0.3×10^8 organisms per mL.

in sterile normal saline. Control mice were injected with 0.1 mL of sterile saline. A total of six mice were used for each isolate. All the animals were observed for signs of infection. Blood smears were prepared from the dead mice and stained with Leishman's stain. Re-isolation of *P. multocida* from heart blood, lung, liver and spleen of the dead mice was carried out on sheep blood agar.

Antibiogram

The antibacterial sensitivity patterns to 15 different antibacterial was done as per the standard single disc diffusion method of Bauer *et al.* (1966).

Isolation of Plasmid DNA

Plasmid DNA was isolated as per the method of Sambrook *et al.* (1989) with minor modifications. Pure culture of *P. multocida* was inoculated in 10 mL of Luria Bertani broth and incubated at 37°C for 8 h. The broth was centrifuged at 8000 x g for 15 min. Resuspended the pellet in 100 µL of TEG buffer containing lysozyme at a concentration of 10 mg mL⁻¹ and was kept on ice for 15 min. To this mixture 200 µL SDS-NaOH was added and gently mixed until the solution became translucent. The tube was further incubated on ice for 15 min. To this 150 µL of 3M sodium acetate was added and kept on ice for another 15 min. The mixture was centrifuged at 12000 x g for 30 min at 4°C. The supernatant was carefully transferred into a fresh Eppendorf tube and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by gentle inversion for 10 min and centrifuged at 10,000 x g for 5 min. The aqueous phase was transferred to a fresh tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently by inversion for 10 min and centrifuged at 10,000 x g for 5 min. The aqueous phase was transferred into a fresh Eppendorf tube, added double the volume of ice-cold ethanol and allowed the plasmid DNA to precipitate at -70°C overnight. The tube was then thawed and centrifuged at 10,000 x g for 15 min. The DNA pellet was washed in 70% ethanol and the final pellet was resuspended in 20 µL of TE buffer. Ribonuclease A (10 mg mL⁻¹) 2 µL was added and incubated at 37°C for half an hour and then stored at -20°C. Plasmid DNA from *Escherichia coli* V517 maintained in the Department of Microbiology was prepared in a similar manner.

Electrophoresis

The isolated plasmid DNA was analysed in 0.8% agarose gels. *Escherichia coli* V517 plasmid DNA was used to ascertain the size of the plasmids. Electrophoresis was carried out at 40V till the dye reached near the bottom of the gel. The DNA fragments were viewed on a transilluminator and photographed using a gel documentation system (Bio-Rad, USA).

RESULTS

Pathogenicity Testing

All the 25 isolates of *P. multocida* as well as the reference fowl isolate were able to kill weaned mice. A concentration of 0.3×10^8 organisms per 1 mL was able to kill mice within 24 h when injected by intra-peritoneal route.

The gross lesions observed in the internal organs of the dead mice were petechiae in the pericardium and congestion of lung, liver and spleen. Blood smear and impression smears from spleen and liver collected from dead mice, on staining with Leishman's stain, revealed the presence of bipolar shaped organisms.

Antibiogram of *P. multocida* isolates indicating the susceptibility and resistance to various antibiotics/antibacterial agents is presented in Table 1.

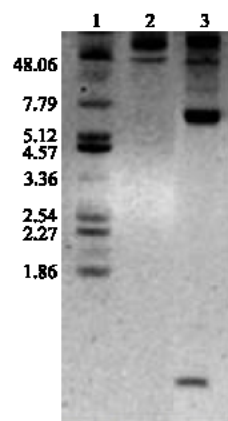
Table 1: Antibigram of isolates

	DP1	2	3	4	5	6	7	8	9	10	11	12	13
Ampicillin	R	R	R	S	S	S	S	S	S	S	R	S	S
Cloxacillin	R	R	S	S	R	R	R	R	R	S	S	S	S
Penicillin	R	R	S	S	S	R	R	S	S	R	S	S	S
Enrofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S
Pefloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	R	R	S	S	S	S	S	S	R	S	S	S
Streptomycin	R	R	R	R	R	R	R	S	R	S	S	S	R
Tetracycline	S	S	S	S	S	S	S	R	S	S	S	S	S
Metronidazole	R	R	R	R	R	R	R	R	R	R	R	R	R
Metronidazole	S	R	S	S	S	S	R	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S	S
Co-trimoxazole	S	S	S	S	R	R	R	S	S	S	S	S	S
Furazolidone	R	R	R	R	S	S	S	R	R	R	S	S	R
Ciprofloxacin	S	S	R	S	S	S	S	S	S	S	S	S	S
Bacitracin	S	S	R	S	S	S	S	S	S	S	S	S	S

Table 1: Continued

	14	15	16	17	18	19	20	21	22	23	24	25	LKO
Ampicillin	S	S	S	S	S	S	S	S	S	S	S	S	R
Cloxacillin	S	S	S	S	S	S	S	S	S	S	S	S	R
Penicillin	S	R	S	S	S	R	S	S	S	S	S	S	R
Enrofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S
Pefloxacin	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	R	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin	R	S	R	R	R	R	R	R	R	R	R	S	R
Tetracycline	S	S	S	S	S	S	S	S	S	S	S	R	S
Metronidazole	R	R	R	R	R	R	R	R	R	R	R	R	R
Metronidazole	S	S	S	S	S	S	S	S	S	S	S	R	R
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S	S
Co-trimoxazole	R	S	S	S	R	R	S	S	S	S	S	S	S
Furazolidone	R	R	R	R	R	R	R	R	R	R	R	S	R
Ciprofloxacin	S	S	R	S	S	S	S	S	S	S	S	R	S
Bacitracin	S	S	S	S	S	S	S	S	S	S	S	S	S

S = Susceptible, R = Resistant

Fig. 1: Plasmid profiles of *P. multocida*, Lane 1 *E. coli* V517, Lane 2 profile I, Lane 3 profile II

Plasmid Profile Analysis

The twenty-five duck isolates could be placed into two plasmid profiles (Fig. 1). Profile II consisted of a single isolate, DP6, which carried four plasmids with molecular sizes of 46.2, 10.9, 6.5 and 0.8 kbp. Of the remaining twenty-four duck isolates three (DP 8, DP 12 and DP 18) did not harbour any plasmids, while the remaining 21 carried a single plasmid each of 46.2 kbp size (Profile I). The reference fowl isolate did not harbour any plasmid.

DISCUSSION

All the field isolates as well as the reference isolates were pathogenic for mice. Sambyal *et al.* (1988) Jayakumar (1998) observed that *P. multocida* of duck origin killed mice within 12 to 24 h post inoculation.

Collins and Woolcock (1976) observed an overwhelming increase in the number of *P. multocida* in visceral organs associated with death in mice, following experimental infection. Similar observations have also been made by Balakrishnan (1998) and Rajalakshmi (2001).

All isolates were sensitive to Enrofloxacin, Pefloxacin and Chloramphenicol. Seven isolates representing 25.92% of the isolates tested were found to be resistant to co-trimoxazole. Moderate sensitivity to co-trimoxazole has been reported by Sambyal *et al.* (1988) and Bhaumik and Dutta (1995). This present study indicates a shift in sensitivity pattern of drugs once considered to be the drug of choice for avian pasteurellosis. Hence it is important to check the indiscriminate use of antibiotics in the treatment of pasteurellosis. Furazolidine and streptomycin showed a sensitivity of only 22.2% while all the isolates were resistant to metronidazole. From this it is evident that the drug of choice for the treatment of duck pasteurellosis in this geographical location is Enrofloxacin, pefloxacin or chloramphenicol.

Of the twenty-five duck isolates, twenty-two (88%) carried plasmids that ranged from 46.2 to 0.8 kbp. Strains of *P. multocida* have been shown to harbour plasmids from 1.3 kbp. (Diallo *et al.*, 1995) to approximately 100 kbp (Hirsh *et al.*, 1989) in size.

Gunther *et al.* (1991) demonstrated that among 28 isolates of *P. multocida* from rabbits, 92% carried plasmids. However, two other studies showed greater variation in the occurrence of plasmids in avian isolates and it varied from 24% (Price *et al.*, 1993) to 70.7% (Hirsh *et al.*, 1985).

Diallo *et al.* (1995) observed that of the 45 avian strains of *P. multocida* studied, twenty strains yielded no plasmids. The remaining twenty-five were placed into two plasmid profiles. Profile I consisted of seven isolates that carried a single plasmid each of 1.3 kbp while the remaining 18 were grouped into profile II that showed two plasmids each of 2.4 and 7.5 kbp

Similar results have also been reported by Shivshankara *et al.* (2000). They identified that 10 out of 12 isolates of *P. multocida* contained plasmids and they were placed into two groups, group one containing seven isolates, carrying a single plasmid and remaining isolates forming group II harbouring two plasmids each.

The isolates DP 8, DP 12 and DP 18 did not harbour any plasmids. However, they were found to be resistant to five, one and four antimicrobial agents respectively out of the 15 antimicrobials tested. Thus correlation between the presence of plasmids and antibiotic resistance could not be ascertained in the present study. Similar observations have been made by Diallo *et al.* (1995) who found that although only 55% of the 45 avian isolates of *P. multocida* carried plasmids, all of them were uniformly resistant to streptomycin, trimethoprim and lincomycin.

Pathogenicity tests conducted in mice revealed that all the isolates, even those which lacked plasmids were pathogenic. Thus this study could not establish any correlation between the presence of plasmids and virulence. These results are in accordance with the observations made by Diallo *et al.* (1995), Balakrishnan (1998) and Shivshankara *et al.* (2000).

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