RESEARCH ARTICLE

Antibiotic Resistance Pattern of *Pasteurella multocida* of Avian Origin

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Abstract

The present study documents the antibiotic resistance phenotypes among 134 strains of *Pasteurella multocida* isolated from chicken, ducks, turkeys, pigeons and geese in India. *P. multocida* type A was found to be predominant capsular type causing fowl cholera. The isolates were resistant to sulphadiazine (90.3%), tetracycline (32%), pefloxacin (20.9%), ciprofloxacin and spectinomycin (18.6%). The sensitivity pattern indicates that the gentamicin was most effective (98.5%), followed by amoxyclav (97.7%), ampicillin (93.3%) and ceftriaxone (94%). Sulfamethoxazole-trimethoprim was found to be effective against 83.5% of the isolates. Erythromycin and enrofloxacin exhibited intermediate sensitivity of 57% and 67.1%, respectively. Also 23.8% isolates were found to be multidrug resistant and was statistically significant ($p<0.05$). The emergence of multidrug resistant strains of *P. multocida* among poultry warrants the judicious use of antimicrobial agents for treating the diseases caused by *P. multocida* in avian spp.

Key words: Antibiotic resistance, India, *Pasteurella multocida*, Poultry.

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Introduction

Fowl cholera is a highly contagious and economically important disease in commercial poultry, backyard poultry, ducks, turkeys and other avian species. The disease is caused by *Pasteurella multocida*, a Gram-negative coccobacilli. The disease is known to occur in per acute, acute and chronic forms resulting in severe economic losses to the poultry industry. Based on the capsular antigen, *P. multocida* is grouped into 5 capsular types A, B, D, E and F. Strains of capsular type A are recognized as the primary cause of fowl cholera, whereas types B, D and F are less frequently associated with disease. The control of the disease is either by vaccination or by antibiotic therapy. Inspite of vaccination of birds, fowl cholera has remained major economic loss to the poultry industry (Victor *et al*., 2016). Although different strains of *P. multocida* are generally susceptible to the majority of the antimicrobials, the incidence of multidrug resistant strains has been widely reported in the last several decades in several countries (Furian *et al*., 2016).

Some antibiotics like sulphonamides, tetracycline, penicillin and chloramphenicol are effective, if administered early. However, the prolonged and indiscriminate use of antibiotics has resulted in the development of resistance among various strains of the organisms (Arora *et al*., 2005) and even multidrug resistant (MDR) forms of *P. multocida* have emerged (Shivachandra *et al*., 2005). Although, several reports have been published on antimicrobial resistance pattern of *P. multocida* isolates in livestock and poultry in several countries, very limited reports are available for Indian isolates. Lack of this antimicrobial resistance surveillance data on *P. multocida* is a major limitation to initiate early and effective antibiotic therapy to diseased birds. For effective treatment and control of pasteurellosis in animals it is necessary to determine bacterial resistance to antibiotics of all classes (phenotypes) and genes that are responsible for bacterial resistance to antibiotics (genetic analysis). Hence, the present study was undertaken to determine the antimicrobial susceptibility pattern of 134 isolates of *P. multocida* of avian origin, which will assist veterinary practitioners in the selection of appropriate antimicrobial agents and to make prudent use of antibiotics for controlling pasteurellosis in birds.

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Materials and Methods

Bacterial Strains

An aliquot of one hundred and thirty four (134) freeze dried cultures of *P. multocida* isolated from different avian species during fowl cholera outbreaks in different parts of India over

a period of 16 years (2000-2016) and maintained in the culture repository of Haemorrhagic Septicaemia (HS) laboratory, Division of Bacteriology & Mycology, Indian Veterinary Research Institute, Izatnagar were used in the study. The *P. multocida* strains included were isolated from chicken (n=75), duck (n=47), emu (n= 4), quail (n= 3), pigeon (n= 2), turkey (n= 2) and geese (n= 1). The details of the isolates are presented in Table 1.

Table 1: Number of isolates of *P. multocida* obtained from different avian species

Species	No. of isolates	Serogroup				
		A	B	D	E	F
Ducks	47	45	1	None	None	1
Emu	4	4	None	None	None	None
Geese	1	None	1	None	None	None
Pigeon	2	2	None	None	None	None
Chicken	75	63	8	4	None	None
Quail	3	3	None	None	None	None
Turkey	2	2	None	None	None	None

Revival and Confirmation of *P. multocida* Isolates

The cultures were revived in brain heart infusion (BHI) broth by incubating at 37 °C for 24 h. The revived broth cultures were then inoculated on 5% sheep blood agar in order to study their morphology and cultural characteristics. The genomic DNA of the isolates was extracted by CTAB method. Later, the pure cultures of the isolates were confirmed by *P. multocida* specific PCR (PM-PCR) using set of primers as described elsewhere (Townsend *et al*., 2001) and capsular typing was done using capsular PCR as described by Townsend *et al*. (1998). The primers used for PM-PCR and multiplex capsular typing are listed in the Table 2. The PCR reaction mixture and cycling conditions for PM-PCR and capsular PCR were same as that of the method described by Townsend *et al*. (1998). The PCR products were subjected to agarose gel electrophoresis using 1.5% agarose (Sigma, USA) and then visualised by UV gel documentation system (Alpha Imager, Germany).

Table 2: List of primers used for PM PCR and Capsular PCR assays

Gene	Primer	Primer sequence (5'-3')	Amplified product size (bp)
KMT1	PM PCR	**F**- ATCCCGCTATTTACCCAGTGC **R**- GCTGTAAACGAACTCGCCAC	460
hyaD-hyaC	capA	**F**- GATGCCAAAATCGCAGTCAG **R**- TGTTGCCATCATTGTCAGTG	1044
bcbD	capB	**F**- CATTTATCCAAGCTCCACC **R**- GCCCGAGAGTTTCAATCC	760
dcbF	capD	**F**-TTACAAAAGAAAGACTAGGAGCCC **R**- CATCTACCCACTCAACCATATCAG	657
ecbJ	capE	**F**-TCCGCAGAAAATTATTGACTC **R**- GCTTGCTGCTTGATTTTGTC	511
fcbD	capF	**F**-AATCGGAGAACGCAGAAATCAG **R**-TTCCGCCGTCAATTACTCTG	851

Antibiotic Sensitivity Test

Antibiotic susceptibility testing was performed by the Kirby-Bauer disc diffusion method as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008). All the isolates were tested for their susceptibility pattern using a panel of 17 different antibiotics (Hi-Media laboratories, Mumbai, India) as specified in Table 3. The diameters of the zone of inhibition surrounding the

Table 3: Antibiotic sensitivity patterns of 134 isolates against 17 different antibiotics

S.No	Antibiotic	Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
1	Ampicillin (10 µg)	9 (6.71)	0 (0)	125 (93.3)
2	Amoxycillin (30 µg)	3 (2.23)	0 (0)	131 (97.7)
3	Chloramphenicol (30 µg)	15 (11.2)	9 (6.71)	110 (82)
4	Ciprofloxacin (5 µg)	25 (18.6)	28 (20.9)	81 (60.4)
5	Cefepime (5 µg)	26 (19.4)	0 (0)	108 (80.5)
6	Cefoperazone (75 µg)	1 (0.74)	16 (11.9)	117 (87.3)
7	Ceftriaxone (30 µg)	5 (3.73)	3 (2.23)	126 (94)
8	Erythromycin (15 µg)	3 (2.23)	60 (44.7)	71 (53)
9	Enrofloxacin (10 µg)	3 (2.23)	41 (30.6)	90 (67.1)
10	Gentamicin (10 µg)	1 (0.74)	1 (0.74)	132 (98.5)
11	Kanamycin (30 µg)	12 (8.95)	40 (29.8)	82 (61.1)
12	Pefloxacin (5 µg)	28 (20.9)	0 (0)	106 (79.1)
13	Streptomycin (10 µg)	15 (11.2)	16 (12)	103 (76.8)
14	Sulfatrimethaxazole (25 µg)	11 (8.2)	11 (8.2)	112 (83.5)
15	Spectinomycin (100 µg)	25 (18.6)	10 (7.46)	99 (73.8)
16	Tetracycline (30 µg)	43 (32)	0 (0)	91 (68)
17	Sulphadiazine (100 µg)	28 (90.3)[a]	0(0)	3 (9.67)

a= only 31 isolates tested against sulphadiazine

antibiotic discs were measured and subsequently matched with the standard zone of inhibition diameters of respective antibiotic disc. The isolates were interpreted as sensitive, intermediately sensitive and resistant based on the interpretative criteria.

Statistical Analysis

Statistical anlaysis was done using SPSS version 16.0 (SPSS Inc, Chicago, Illinois, USA). Chi-square test was employed to evaluate whether there is any significant difference between the percentage of isolates resistant to different antibiotics. A value of $p<0.05$ was considered to be statistically significant.

Results and Discussion

In the current study, PM-PCR correctly identified all isolates as *P. multocida* by the presence of 460 bp band (Fig. 1), which is consistent with other studies (Dey *et al*., 2007; Kumar *et al*., 2009). In multiplex capsular PCR assay, 119 (88.8%) isolates were found to be capsular type A and produced an amplicon of ~1044 bp, 10 (7.46%) isolates were of type B and yielded an expected amplicon of ~ 760 bp, 4 (3%) isolates belonged to type D and yielded an amplicon of 657 bp, 1 (0.74%) duck isolate belonged to capsular type F with an amplicon of ~ 851 bp (Fig. 2, 3) and none of the isolates belonged to type E. The amplification of ~ 1044 bp, ~760 bp, ~ 657 bp and ~ 851 bp products using capsular primers is specific for the capsular types A, B, D and F, respectively, of *P. multocida* (Townsend *et al*., 1998). Capsular typing revealed that vast majority of the isolates belongs to type A (88.8%) compared to other capsular types. One study reported that 68% of the *Pasteurella multocida* isolates recovered from cases of fowl cholera in England and Wales over a 13-year period were of capsular type A, 14% were type F, 5% were type D, 4% were type B and 9% were untypable (Davies *et al*., 2003). In another study, 85.71% and 14.28% of the isolates from chicken were of type A and type D, respectively (Deka *et al*., 2017). Earlier studies (Rimler and Rhoades, 1987) also recognized A, B, D and F serogroups in avian strains of *P. multocida*.

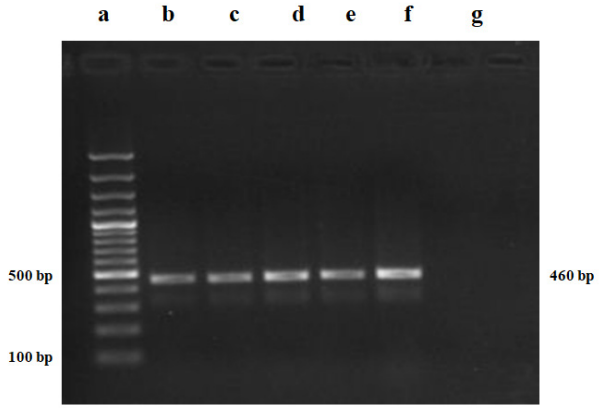

Fig 1: PMPCR of the isolates, a 100 bp ladder, b - f: isolates, g: Negative control

Generally, *P. multocida* isolates are susceptible to most of the presently available commercial antimicrobial agents (Rigobelo *et al*., 2013). Also, the strains with different antimicrobial resistance phenotypes are always present in animal populations. Their number and types may vary according to host origin, geographical location and the previously applied antimicrobial therapy in the given population. On antibiogram of the isolates, the most prevalent phenotypes detected were resistant to sulphadiazine (90.3%), tetracycline (32%), ciprofloxacin and spectinomycin (18.6%). The highest intermediate resistance was observed against erythromycin (44.7%) and enrofloxacin (30.6%). The resistance pattern of isolates for other antimicrobial agents is presented in Table 3. None of the antimicrobial agent was found to be 100% effective against the isolates. Resistance to cefepime, a fourth generation cephalosporin was also remarkable. These antibiotics are commonly used for treatment of various infectious diseases in livestock and poultry in India and the excessive use of these drugs could be the reason for the development of resistance due to the selective pressure imposed by these antibiotics in the isolates. This report is similar to the findings of Shivachandra *et al*. (2005), who observed 100% resistant isolates to sulphadiazine. Similarly, the studies in Hungary (Sellyei *et al*., 2009) and Nigeria (Dashe *et al*., 2013) observed increased resistance of *P. multocida* strains isolated from poultry to sulphonamides, macrolides and tetracyclines. The current findings as well as those of other workers (Prabhakar *et al*., 2012; Balakrishnan and Roy, 2012) indicated that the antibiotics sulphonamides, tetracyclines and macrolides may not be effective in controlling fowl cholera in the region.

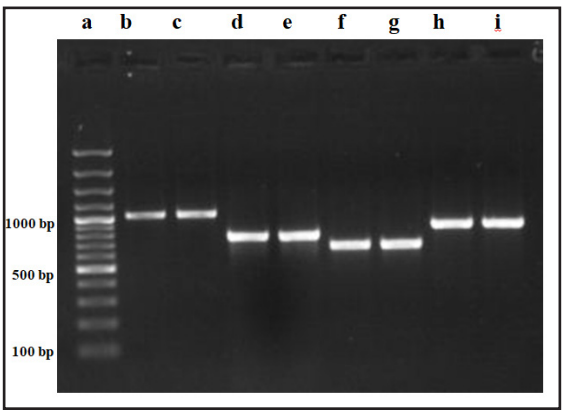

Fig 2: Capsular PCR of the isolates, a 100 bp ladder, b and c: Type A (1044 bp) d and e: Type B (760 bp) f and g: Type D (657 bp) h and i: Type F (851 bp)

The sensitivity pattern indicated that the gentamicin was most effective (98.5%), followed by amoxycillin (97.7%), ampicillin (93.3%) and ceftriaxone (94%). Sulfatrimethaxazole was found to be effective against 83.5% of the isolates. The majority of the strains exhibited intermediate sensitivity to other tested antimicrobials. The present findings also corroborated with the findings of Furian *et al*. (2016), who reported gentamicin and amoxycillin as effective

antimicrobials against avian pasteurellosis. In contrast to our findings, high resistance of *P. multocida* to ampicillin, amoxicillin/clavulanic acid and gentamicin was documented (Victor *et al*., 2016). This might be due to the difference in geographical area and increased extent of exposure of these antimicrobial agents to poultry. Also, antimicrobial resistance of *P. multocida* varies according to the host animal species, time and geographical origin of the animals (Naz *et al*., 2012).

In addition it was observed that 82 (61.2%) isolates were resistant to at least one antimicrobial agent tested and 32 (23.8%) isolates were found to be multidrug resistant (resistant to 3 to 10 antibiotics) ($p<0.05$). The numbers of isolates resistant to one, two and three or more antibiotics were found to be in the order 30, 20 and 32, respectively. In a study in Southern Brazil (Furian *et al*., 2016) 19.64% of the poultry strains were resistant to 3 or more drugs in different categories. The uncontrolled and widespread use of antibiotics for growth promotion and for prophylactic treatment is resulting in the emergence of resistant strains. Due to the emergence of multidrug resistant *P. multocida* in poultry, the antibiotics should be used preferably after performing antibiotic sensitivity test in cases of fowl cholera. If antibiogram is not feasible, gentamicin, amoxycillin, ampicillin and ceftriaxone must be given preferential consideration over other antibiotics particularly sulphadiazine, trimethoprim, erythromycin, enrofloxacin, and tetracycline for fowl cholera in India.

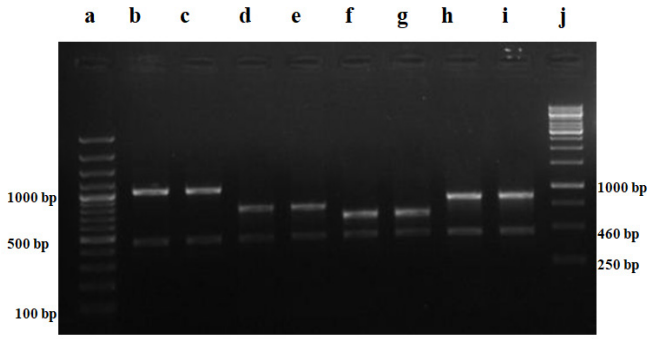

Fig 3: Multiplex PCR of the isolates, a 100 bp ladder, j 1kb ladder, b and c: Type A, d and e: Type B, f and g: Type D, h and i: Type F

Conclusion

In findings of the present study revealed that, *P. multocida* capsular type A was more frequently associated with avian pasteurellosis in India and the moderate prevalence of multidrug resistant strains and the associated antimicrobial genes in the isolates warrants the need for the more prudent use of antimicrobials. The high resistance of isolates to sulphonamides, tetracyclines, and ciprofloxacin has highlighted that prevention and therapeutic effect on avian strains of *P. multocida* should no longer be expected from these antibiotics in India and gentamicin, amoxycillin could be used as drugs of choice to control fowl cholera.

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