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Capsular typing and virulence gene profiling of *Pasteurella multocida* isolated from chickens

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Abstract
A study was conducted to identify the prevalent capsular types of *Pasteurella multocida* in chicken from different parts of Assam, India. Screening of 153 samples, comprising of tracheal swab (71), lungs (52), liver (20) and heart blood (10) revealed 14 samples to be positive for *P. multocida*, yielding an equal no. of isolates. All the isolates were confirmed to be *P. multocida* by pm-PCR. Among the isolates, 12 were identified as capsular type A (85.71%), while the remaining 2 (14.28%) isolates as type D. Isolates were found to possess virulence associated genes in different combinations, irrespective of their capsular types. All the 14 isolates were found to possess *omp*H gene, while 10 could reveal presence of *omp*A87 (71.42%) gene. Presence of *fim* A was observed in 9 (64.28%) isolates, while *ptf*A and *nan*B could be detected in 6(42.85%) isolates. *pfhA* and *tbp* gene could be detected in 5 (35.0%) of the isolates, while 3 (21.0%) isolates revealed presence of *hgb* gene. Distribution of virulence associated gene was found to be equal in the *P. multocida* isolates of both apparently healthy and diseased chickens.

Keywords: *Pasteurella multocida*, pm-PCR, cap-PCR, Capsular type.

Introduction
Fowl cholera is a contagious and economically important disease of poultry, particularly of chicken, turkeys, ducks and geese (Glisson *et al*. 2003) [9]. Acute form of the disease is usually associated with septicaemia, resulting high morbidity and mortality. *P. multocida* capsular type A is reported to be predominantly associated with most severe form of fowl cholera (Boyce and Adler 2000) [3]. Research on the molecular mechanisms by which *P. multocida* can survive and multiply within a host resulting disease production are poorly understood. Various genes associated with numerous factors, like amino acid metabolism, and iron transport and nucleotide synthesis (Homchampa *et al.* 1997 and Fuller *et al.* 2000) [11] [7], capsules (Boyce and Adler 2000) [3], hemagglutinins and hemolysins (Fuller *et al.* 2000) [7], toxins in a few toxin-producing strains (Lax and Grigoriadis 2001) [14] may be associated directly or indirectly with pathogenesis of avian pasteurellosis. In spite of development of molecular strategies targeting virulence associated genes in *P. multocida*, the distribution of those genes in different hosts is poorly studied. Considering all, a study was undertaken to investigate the prevalence of genes associated with virulence in the isolates of *P. multocida* of bird origin.

Materials and Methods
Isolation and Identification: A total 153 nos. of clinical sample comprising of lungs (52), tracheal swabs (71), livers (20) and heart bloods (10) were collected from apparently healthy (73) and diseased/dead chicken (80). Isolation was done by inoculating the samples into sheep blood agar plate (5.0% v/v), as per the method of Collins and Lyne (1970) [4]. Isolated colonies, suggestive of *P. multocida* were identified on the basis of typical staining characteristics, cell morphology and biochemical characteristics. Confirmed colonies of *P.multocida* were further subjected for molecular confirmation and their capsular type.

Molecular confirmation and capsular typing: All the *P. multocida* isolates were tested for molecular confirmation, targeting the gene encoding for species specific *P. multocida* KMT1 fragment by Polymerase Chain Reaction (pm-PCR), as per the method of Townsend *et al*. (1998) [30]. Isolates, bearing KMT1 gene were further subjected for molecular typing by cap-PCR (Townsend *et al*. 1998 and 2001) [30, 28].

DNA was extracted from each isolates of *P. multocida* as per the method described by Titball *et al.* (1998) [29]. Extracted DNA templates of *P. multocida* isolates were subjected to species specific simplex pm-PCR for reconfirmation by using primer pair KMT1T7- KMT1SP6. All the confirmed *P. multocida* isolates were tested for their capsular types by cap-PCR, targeting *hyaD-hyaC* (TypeA), *bcbD* (Type B) and *bcbF* (Type D). Both pm-PCR and cap-PCR were carried out by using previously reported primers (Table1). The PCR reaction was carried out in 25 µl reaction mixture, comprising of 3 µl DNA template in 12.5 µl master mix (2x), 0.125 µl of each species specific primer (forward and reverse) and nuclease free up to 25 µl. The reaction mixture was subjected to amplification in a thermocycler (Applied Biosystem, USA) with initial denaturation (95°C) for 4 min, followed by 30 cycles of denaturation at 95° C for 45 sec, annealing (55°C) for 45 sec, extension (72° C) for 45 sec. Final Extension was done at 72°C for 10 min. The amplified products were electrophoresed in 1.5 percent agarose gel with 1x Tris Acetate EDTA (TAE) buffer at 80 volt for 1 hour with ethidium bromide stain. DNA fragments were visualized with UV light by Gel documentation system (Bio-Imaging system Mini Lumi, Israel).

Virulence gene profiling: Isolates were further screened for gene profiling, in respect to six virulence associated genes, encoding for outer membrane proteins (ompH and ompA87),colonization and adhesion related proteins (ptfA and fimA) and iron binding proteins (hgbB and tbpA). Extracted DNA was subjected to simplex PCR using previously reported primers (Table 1) for respective genes. The amplification was carried out in 25 µl reaction mixture containing 12.5 µl of master mix (2x) 0.5 µl of each primer, 3 µl of template DNA and nuclease free water to make the final volume of 25 µl. The PCR reaction was performed in a thermocycler with the reported PCR condition (Table 2).

Table 1: Primer sequences for respective genes

Gene	Sequence (5-3)	Size (bp)	Reference
kmt	ATCCGCTATTTACCCAGTGG-3'	460	Towsend *et al.* (1998) [30]
	GCTGTAAACGAACTCGCCAC-3'		
*hyaD-hyaC (*Type A)	TGCCAAAATCGCAGTCAG	1044	Towsend *et al.* (1998) [30]
	TTGCCATCATTGTCAGTG		
bcbF (TypeD)	TTACAAAAGAAAGACTAGGAGCCC	657	Towsend *et al.* (2001) [28]
	CATCTACCCACTCAACCATATCAG		
bcbD (Type B)	CATTTATCCAAGCTCCACC	765	Towsend *et al.* (2001) [28]
	GCCCGAGAGTTTCAATCC		
ompH	ACTATGAAAAAGACAATGGTAG	1000	Luo *et al.* (1997) [16]
	GATCCATTCCTTGCAACATATT		
ompa87	GGCAGCAGCAACAGATAACG	838	Ewers *et al.*(2006) [6]
	TGTTCGTCAAATGTCGGGTGA		
ptfA	AATGCCAGTCCACTCGTT GT	1088	Sellyei *et al.* (2010)
	CGTCCTGAGGCAAGCGTGTT		
fimA	CCATCGGATCTAAACGACCTA	866	Tang *et al. (*2009) [27]
	AGTATTAGTTCCTGCGGGTG		
tbpA	TGGTTGGAAACGGTAAAGC	728	Shayegh *et. al.* (2008) [23]
	TAACGTGTACGGAAAAGCC		
hgbB	TCTTTGAGTACGGCTTGAC	540	Shayegh *et. al.* (2008) [23]
	CTTACGTCAGTAACACTC		

Table 2: Thermal cycler conditions for virulence genes detection

PCR step	Virulence associated Gene						No. of Cycles
	ompa87	***ompH***	***ptfA***	***fimA***	***tbpA***	***hgbB***	
Initial denaturation	94°C, 3min	94°C, 3 min	95°C, 5 min	95°c, 5 min	94°C, 3 min	95°C, 5 min	
Denaturation	94°C, 30 sec	94°C, 30 sec	95°C, 30 sec	95°C, 30 sec	94°C, 30 sec	94°C, 30 sec	30
Annealing	57°C, 30 sec	57°C, 30 sec	50°C, 30 sec	50°C, 30 sec	55°C, 30 sec	55°C, 30sec	
Extention	72°C, 1min	72°C, 1 min	72°C, 1 min	72°C, 1 min	72°C, 60 sec	72°C, 50 sec	
Initial denaturation	94°C, 3 min	94°C, 3 min	95°C, 5 min	95°C, 5 min	94°C, 3 min	95°C, 5 min	
Denaturation	94°C, 30 sec	94°C, 30 sec	95°C, 30 sec	95°C, 30 sec	94°C, 30 sec	94°C, 30 sec	
Final extension	72°C,10 min						
Reference	Ewers *et al.* (2006) [6]	Luo *et al.* (1997) [16]	Sellyei *et al.* (2010) [22]	Tang *et al.* (2009) [27]	Shayegh *et. al.* (2008) [23]	Shayegh *et. al.* (2008) [23]	

Results and Discussion

The present investigation revealed isolation of 14 (9.15%) nos. of *Pasteurella multocida* isolates from 153 nos. of clinical sample (Table 3), of which six were isolated from apparently healthy and eight were from diseased/dead chicken. Highest percentage of isolation was recorded from

lung (15.38%), followed by heart blood (10.0%) and tracheal swab (7.04%). None of the liver samples revealed presence of *P. multocida.* Recovery of *P. multocida* from apparently healthy and from diseased/dead chicken was also previously reported by Masdooq *et al.* (2008) [17] and Prabhakar *et al.* (2012) [20]. Although *P. multocida* remains as commensal in the airways of birds, frequency of the organism invariably increases in diseased or dead birds, resulting an increase frequency of isolation of *P. multocida* from dead/diseased bird. Being a commesal of upper respiratory track and primary involvement of lungs during infection, rate of recovery of *P. multocida* is found to be highest from lungs of chicken, irrespective of health status (Stephan *et al*. 1995and Altrock 1998) [25][1]. Isolation of *P. multocida* from trachea and heart blood indicates involvement of respiratory track followed by systemic infection caused by *P. multocida* in chicken (Muhairwa *et al.* 2000 and Borah 2006) [19][2].

Table 3: Isolation of *Pasteurella multocida* from apparently healthy and diseased/dead birds

Nature of sample	Heath status of the birds	No. of sample examined	No. of sample positive for *P. multocida*	No. of *P. multocida* isolates recovered
Lung	Apparently Healthy	27	1	8 (15.38%)
	Diseased/Dead	25	7	
Tracheal swab	Apparently Healthy	46	5	5 (7.04%)
	Diseased/Dead	25	0	
Heart blood	Dead	10	1	1 (10%)
Liver	Dead	20	0	0 (0.00%)
Total		153	14	14 (9.15%)

All 14 *P. multocida* isolates were confirmed by pm- PCR with an amplified PCR product of 460 bp size (Fig.1) which has been reported to be confirmatory for identification of *P. multocida* (Townsend *et al.* 1998 and Prabhakar *et al.* 2012) [30, 20]. It can also be opined from the previous study that other bacterial contamination does not affect specificity of pm-PCR based identification of *P. multocida* isolates. Majority (12) of the *P. multocida* isolates were of capsular type A, while the rest two isolates were identified as capsular type D, with amplified products size of 1044 bp and 657 bp respectively (Fig. 2 and 3). The capsular type A was found to be equally distributed among the apparently healthy and diseased/dead chicken (6 each), while isolation of capsular type D was recorded only from diseased/dead birds (Table 4). Association of *P. multocida* type A and D as observed in the present investigation with pasteurellosis in chicken is well established (Sotoodehnia *et al.* 2004 and Mohamed *et al.* 2012) [24][18]. Predominance of serotype A over serotype D in chicken, irrespective of health status was also previously observed by Jabbari *et al.* (2006) [12] and Mohamed *et al.* (2012) [18].

Diversity was observed among the *P. multocida* isolates, in respect to the distribution of virulent genes (Table 4). All the isolates (typeA) were found to possess *omp*H gene (Fig. 4), while detection of *ompA87, ptfA* and *fimA* could be possible in 10 (71.42%), 6 (42.85%) and 9 (64.28%) isolates, respectively. The genes associated with iron-binding proteins, tbp A and hgbB were detected in 35.0 and 21.0 percent of the isolates, respectively (Fig. 5-9). Both the *P. multocida* type D, isolated from diseased/dead chicken were found to possess *omp*H, *ptf*A, *fim*A and *tbp*A, while *omp*A87 and *hgb*B were recorded only in one isolates. Five of the six *ompH* bearing *P. multocida* type A isolated from diseased/dead chicken were found to be positive for *omp*A87 gene, while 4 isolates revealed presence of *fim*A gene. *ptf*A, *hgb*B and *tbp*A gene could be observed only in two of the capsular type A of *P. multocida* isolated from diseased/dead chicken. However, comparatively a less no. of *P. multocida* isolates type A, recovered from apparently healthy chicken could reveal presence of *omp*A87 (4), *fim*A (3), *ptf*A (2) and *tbp*A (1). None of the *P. multocida* isolates type A could show presence of *hgb*B gene.

Detection of *omp*H gene in the *P. multocida* isolates reveals the presence of 37.5-kDa protein H in the envelope of *P. multocida*. Considering the high immunogenicity and cell surface exposed protein, the ompH is opined to be a suitable candidate for vaccine development (Lugtenberg *et al.*1986). Detection of *oma* 87 indicates presence of an additional protective surface protein in *P. multocida* isolates. The oma87 protein is reported to be belonged to the omp85 family (Ruffolo and Adler 1996) [21].The oma87 can be considered to be a protein of interest in the subunit vaccine development, because of its established role in *P. multocida* infection (Tabatabai *et al.* 2008) [26]. Prevalence of the *ptf*A gene (type 4 fimbriae) and *fim*A (fimbriae) found in majority of the *P. multocida* isolates indicates their association with fixation of bacteria cell to the surface of the host epithelial cells (Ewers *et al.* 2006) [6]. TbpA protein is reported to be responsible for extraction of iron from transferrin. TbpA is an integral, TonB-dependent OM protein with large surface exposed loops for binding and forcing the separation of iron from the transferrin (Krewulak and Vogel 2008) [13]. In comparison to the bovine strains of *P. multocida* strains that utilise iron from bovine transferrin, avian strains are unable to capture iron from any transferrin molecules including those from avian source (Hatfaludi *et al.* 2010) [10], which might be the explanation for the low frequency of *tbp*A gene in the chicken isolates of *P. multocida* during the present study. Among the known haem receptors, a haemoglobin-binding protein of 113.5 kDa, designated as HgbB was first identified by Cox *et al.* (2003) [5]. The iron acquisition related gene, *hgb*B expressed on outer membrane of *P. multocida* has been reported to be bind with haemoglobin and the resulting haemoglobin complex. High prevalence of *hgb*B gene in chickens as observed by Ewers *et al*. (2006) [6] indicated its crucial role in the pathogenesis of pasteurellosis in chicken. Contrary to that, detection of *hgb*B gene was recorded less frequently in the isolates of pig origin (Furian *et al.*2013) [8].

The present study could not reveal any correlation between the presence of different virulence associated genes with the diseases caused by *P. multocida.* A large samples size, including isolates from both healthy and diseased animals is necessary to further evaluate this hypothesis.

Table 4: Detection of virulence genes of *P. multocida* isolates by simplex PCR.

Health status	Serotype	No. of isolates tested	No. of isolates positive for virulence gene					
			ompH	*ompA 87*	*ptfA*	*fimA*	*hgb*B	*tbpA*
Apparently Healthy	A	6	6	4	2	3	0	1
Diseased/Dead	A	6	6	5	2	4	2	2
Diseased/Dead	D	2	2	1	2	2	1	2
Total		14	14	10	6	9	3	5

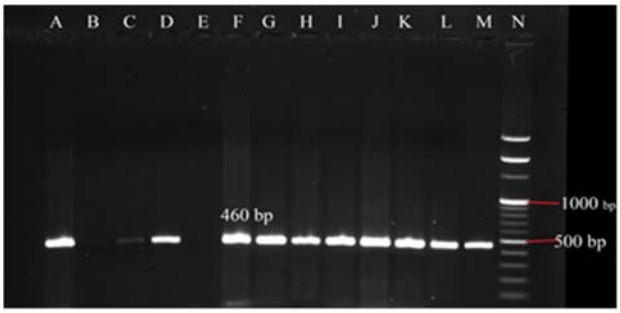

Fig 1: *P. multocida* specific pm-PCR showing an amplified product of 460bp. N = 100 bp ladder, A = Positive control, B and E = Non template control, C, D, F-M= Field isolates

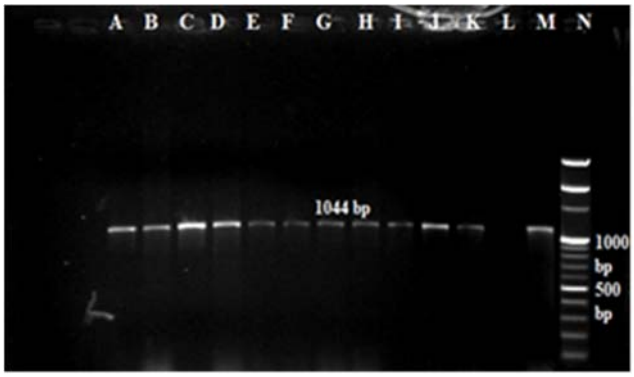

Fig 2: *P. multocida* type specific cap-PCR showing an amplified product of 1044 bp of capsular type A. N= 100 bp ladder, M= Positive control, L= Non template control, A-K= Field isolates type A

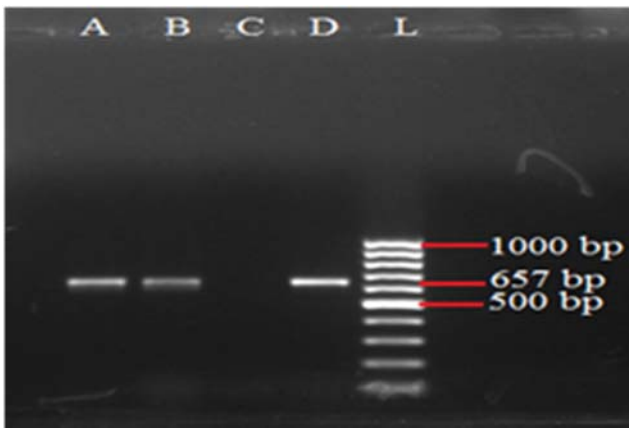

Fig 3: *P. multocida* type specific cap-PCR showing an amplified product of 657 bp of capsular type D. L=100 bp ladder, D= Positive control, C= Non template control, A and B= Field isolates for type D

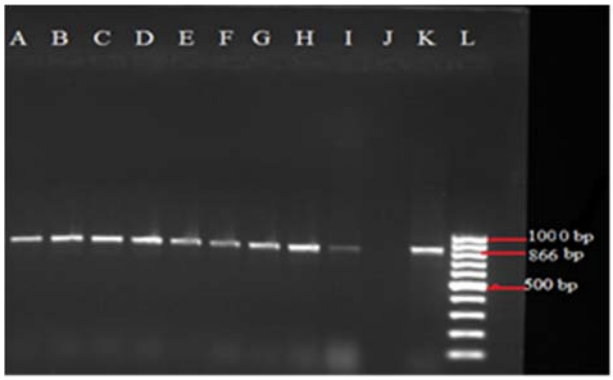

Fig 4: PCR amplified *omph* gene of *P. multocida* with a product size of 1000bp in agarose gel electrophoresis. L= 1kbp ladder, A= Positive control, B= Non template control, C-J= Field isolates

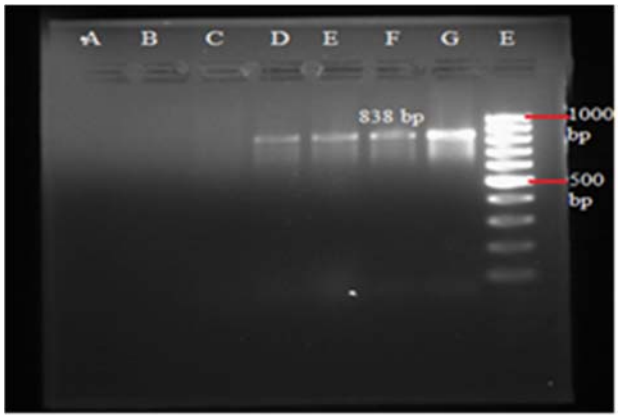

Fig 5: PCR amplified *ompa* 87 of *P. multocida* with a product size of 838 in agarose gel electrophoresis. L=1kbp ladder, G=Positive control, C-F= Field isolates

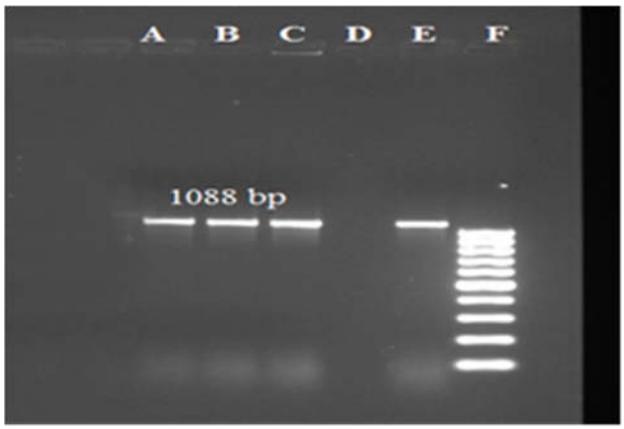

Fig 6: PCR amplified *ptfA* gene of *P. multocida* with a product size of 1088 bp in agarose gel electrophoresis.F= 100 bp ladder, E= Positive control, D= Non template control, A-C= Field isolates

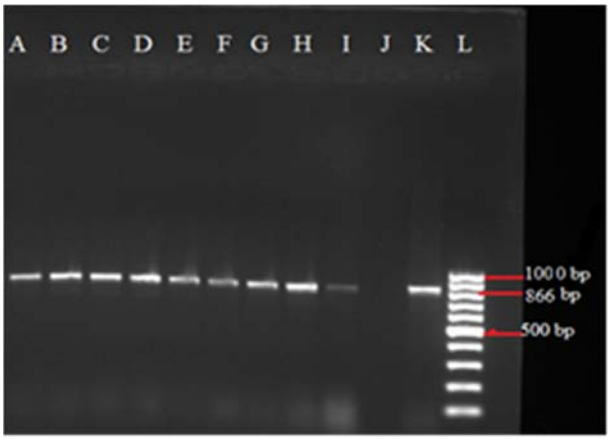

Fig 7: PCR amplified *fimA* gene of *P. multocida* with a product size of 866 bp in agarose gel electrophoresis. L= 100 bp ladder, K= Positive control, J= Non template control, A-I= Field isolates

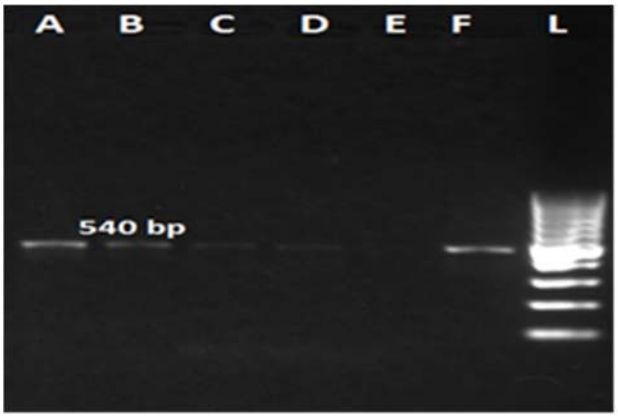

Fig 8: PCR amplified *hgb* gene of *P. multocida* with a product size of 540 bp agarose gel electrophoresis. L= 100 bp ladder, F=Positive control, E= Non template control, A-D= Field isolates.

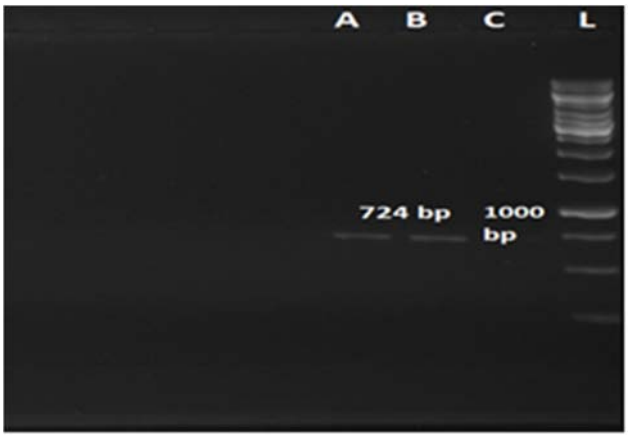

Fig 9: PCR amplified *tbp* gene of *P. multocida* with a product size of 724 bp agarose gel electrophoresis. L= 1kb ladder, B=Positive control, C= Non template control, A= Field isolates.

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