

Isolation, identification, characterization and antibiogram of *Pasteurella multocida* isolated from pigs in Mizoram with special reference to progressive atrophic rhinitis

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Abstract

Aim: Isolation and identification of *Paturella multocida* from pigs of Mizoram with or without clinical lesions of progressive atrophic rhinitis (PAR) followed by their detection, capsular typing and screening for *toxA* gene.

Materials and Methods: Four hundred nasal swabs sampled from pigs were collected from 6 different districts of Mizoram. Swabs were processed for detection and isolataion of *P. multocida* by PCR and traditional bacteriological assays. Isolates were subjected for multiplex PCR for capsular typing, detection of *toxA* gene and characterization by RAPD-PCR. Isolates were also tested for antimicrobial sensitivity profile by disc-diffusion method.

Results: A total of 21 swabs were found to be positive by *P. multocida* species specific PCR (PM-PCR) with an amplicon of 460 bp, of which *P. multocida* could be isolated from 15 swabs (3.75%). All the isolates were grouped under capsular type A (n=9) and D (n=6) by multiplex PCR. All the isolates were found to be negative for *toxA* gene by PCR, indicating the isolates as non-toxigenic. Isolates under similar capsular type provided a unique banding pattern by RAPD-PCR, whereas no discrimination was observed between serotypes.

Conclusion: Although, no isolates exhibited *toxA* gene by PCR, isolataion of *P. multocida* of capsular type A and D may be an indication of their possible role in PAR in pigs of Mizoram.

Keywords: Mizoram, PAR, *Pasteurella multocida*

Introduction

Progressive atrophic rhinitis (PAR) is a widely prevalent, upper respiratory tract disease of swine characterized by sneezing, snuffling, nasal discharge, epistaxis, growth retardation, reduction in feed utilization, degeneration and atrophy of the nasal turbinate bone leading to visible distortion and shortening of the snout [1]. Moreover, it has been recognized as a contributor to debilitating and fatal porcine pneumonia for at least 120 years and continues to be sustained, unabated with high prevalence in different part of the world [2]. Toxigenic strains of *P. multocida* (both capsular type A and D) alone, or combined with environmental factors like dust, ammonia and other physical or chemical irritants are the primary causes of atrophic rhinitis [3].

Livestock plays an important role in this hilly state of Mizoram, as crop production is still traditional. Amongst the livestock, pig is most important and almost every family rears pig as backyard venture. Since the pig rearing is an important sector in rural economy in this region, a better understanding of PAR and its impact on animal husbandry and economy is essential. Studies on PAR should provide a foundation

for induction of the experimental disease, development of improved diagnostics, development of effective therapeutic/ prophylactic pharmaceutical approaches and development of immunoprophylactic products for effective control of the disease.

There is paucity of information regarding PAR in pigs in India, particularly in North Eastern Region. Therefore, the present study was conducted for isolation and identification of *P. multocida* from nasal cavities of pigs of Mizoram with or without clinical lesions of PAR followed by their detection, capsular typing and screening for *toxA* gene by PCR based assays.

Materials and Methods

Sampling: 400 nasal swabs were collected from pigs of different age groups of either sex from 6 districts of Mizoram during June 2010 to May 2011. Of the 400 pigs, 9 were showing respiratory distress as a suspected case of atrophic rhinitis. Samples were collected from local household pigs as well as from organized pig farms.

Detection of *P. multocida* by PCR assay: Each swab was grown in Brain-Heart Infusion (BHI) broth overnight at 37°C. After incubation, 1 ml of bacterial suspension was centrifuged at 7084 g. The bacterial pellet was washed thrice with sterile normal saline solution (NSS) (0.85% w/v) and resuspended in 300 µl

Table-1. Oligonucleotide primers used in the present study

Sr. No.	Name of Primers	Sequence (5'-3')	Expected amplicon size (bp)	Reference
1	KMT1T7-F	ATCCGCTATTTACCCAGTGG	460	17
2	KMT1SP6-R	GCTGTAAACGAACTCGCCAC		17
3	CapA-F	TGCCAAAATCGCAGTCAG	1044	17
4	CapA-R	TTGCCATCATTGTCAAGT		17
5	CapB-F	CATTTATCCAAGCTCCACC	760	17
6	CapB-R	GCCCGAGAGTTTCAATCC		17
7	CapD-F	TTACAAAAGAAAGACTAGGAGCCC	657	17
8	CapD-R	CATCTACCCACTCAACCATATCAG		17
9	CapF-F	AATCGGAGAACGCAGAAATCAG	851	17
10	CapF-R	TTCCGCCGTCAATTACTCTG		9
11	toxA-F	TACTCAATTAGAAAAAGCGCTTTATCTTCC	1230	9
12	toxA-R1	TCCAGTAATTTGTCTGTATTTTATCAAAT		9
13	toxA-R2	TCTACTACAGTTGCTGGTATTTTTAAATAT		10
14	RAPD-PCR	GTGGTGGTGGTGGTG		

Table-2. PCR based screening and isolation of *Pasteurella multocida* from nasal swabs collected from 6 different districts of Mizoram.

Sl. No.	Place of collection	Samples collected	PM- PCR Screening Results	<i>P. multocida</i> isolated	Capsular types
1	Aizawl	185	9	6	A(1); D(5)
2	Lunglei	97	3	3	A(2); D(1)
3	Kolasib	40	2	2	A(2)
4	Serchhip	40	2	2	A(2)
5	Champhai	38	5	2	A(2)
	Total	400	21	15	

of sterile nuclease free water and boiled in a water bath for 10 minutes followed by immediate chilling. The supernatant was used as template DNA for PCR. PM-PCR assay was conducted for the detection of *P. multocida* as described earlier [4].

Mouse inoculation: Each PM-PCR positive culture was injected intraperitoneally to Swiss albino mice (0.2 ml/mice) and observed for 72 hrs. The heart blood and spleen were collected aseptically during postmortem examination and were inoculated on 5% sheep blood agar plate. Plates were incubated at 37°C for overnight. *P. multocida* were isolated and identified using standard bacteriological methods.

Capsular typing of *P. multocida* isolates by multiplex PCR assay: All the isolates were subjected to multiplex PCR assay to determine their capsular group. Each PCR reaction mixture (25 µl) containing the following: each primer (capsular group A, B, D, E and F; Table-1) with concentrations of 3.2 µM, 200µM of each dNTPs, 1x PCR buffer, 1.5 mM MgCl₂, 1 U of Taq DNA polymerase and 4 µl of template DNA. The following standard cycling procedure was used for DNA amplification: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 6 min. The standard reference strain of *P. multocida* serotype B:2 was included as a positive control and the negative control had no template DNA.

Detection of *toxA* gene by *toxA* gene specific PCR assay: *toxA* gene specific PCR was performed [5] by a colony touch PCR mixture (25 µl) containing 3.2 µM of each primer, 200µM of each dNTPs, 1x PCR buffer, 1.5 mM MgCl₂ and 1 U of Taq DNA polymerase. The following standard cycling procedure was used for DNA amplification: initial denaturation at 95°C for 5

min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 6 min.

Randomly amplified polymorphic DNA-PCR: RAPD-PCR [6] reaction mixture (25 µl) was prepared using 1X Dream Taq buffer (with 30 mM MgCl₂), dNTPs (0.2 mM each), RAPD primer (3.5µM), 2.0 U of Taq DNA polymerase and 4.0 µl of template DNA. The reaction mixture was subjected to DNA amplification by following cycling conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 38°C for 1 min and extension at 72°C for 1 min. It was followed by a final extension at 72°C for 6 min.

Amplified products were separated by agarose gel electrophoresis (2% agarose in 0.5 x Tris-borate-EDTA buffer) at 5 V/cm for 2 h and stained with ethidium bromide (0.5 µg/ml). A standard molecular marker (100 bp DNA ladder) was included in each gel. DNA fragments were observed by ultraviolet transilluminator and photographed by gel documentation system (Alpha Imager, Germany).

Antimicrobial drugs sensitivity assay of *P. multocida*: All the isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method [7] using 14 commercially available antimicrobial agents.

Results

PCR based screening of nasal swabs: Of the 400 nasal swabs, 21 animals including the 9 PAR suspected animals, were found to be positive by PM-PCR for a product of 460 bp, which is specific for *P. multocida*. Of the 21 positive samples, 9, 3, 2, 2 and 5 were sampled from Aizawl, Lunglei, Kolasib, Serchhip and Champhai district, respectively (Table-2 and Fig-1).

Isolation and identification of *P. multocida*: A total of

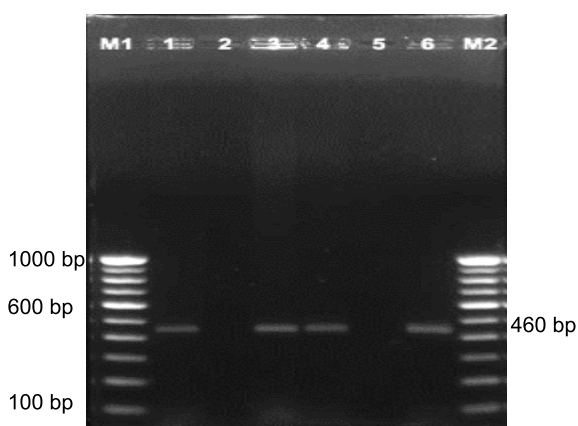


Figure-1. Detection of *Pasteurella multocida* by species specific PCR

Lane M1 and M2: 100 bp DNA ladder, Lane 1: Positive control, Lane 2: Negative control, Lane 3: Sample no. Pm 5, Lane 4: Sample no. Pm 10, Lane 5: Negative sample and Lane 6: Sample no. Pm 15.

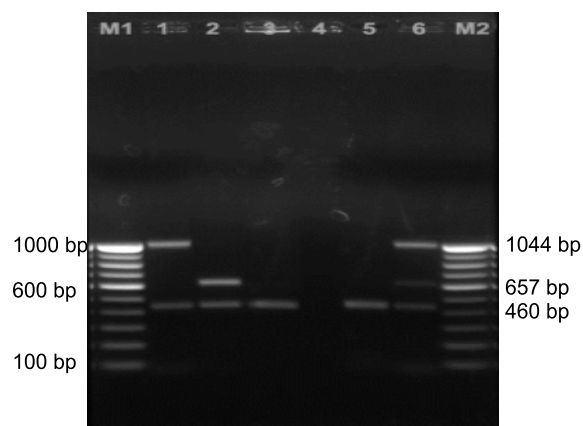


Figure-2. Capsular typing of *Pasteurella multocida* by multiplex PCR

Lane M1 and M2: 100 bp DNA ladder, Lane 1: Capsular type A, Lane 2: Capsular type D, Lane 3: *P. multocida*, Lane 4: Negative control, Lane 5: *P. multocida* and Lane 6: Positive control for capsular type A and D.

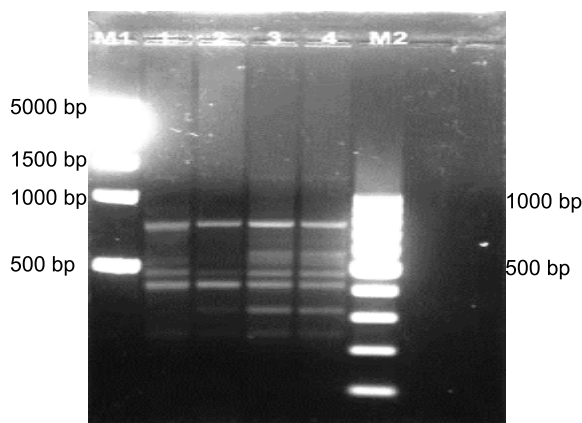


Figure-3. RAPD-PCR analysis of *Pasteurella multocida* isolates.

Lane M1: 500 bp DNA ladder, Lane M2: 100 bp DNA ladder, Lane 1 and 2: Representative *P. multocida* isolates of Capsular type D, Lane 3 and 4: Representative *P. multocida* isolates of Capsular type A.

15 *P. multocida* were isolated and identified and designated as Pm1 to Pm15 (Table-1). *P. multocida* was isolated from all the 9 clinically suspected PAR pigs. Out of 391 apparently healthy pigs, *P. multocida* was isolated from only 6 pigs.

Capsular typing of *P. multocida* by multiplex PCR assay: Of the 15 *P. multocida* isolates 9 (60%) and 6 (40%) were identified as capsular type A (1044 bp) and capsular type D (657 bp), respectively (Table-1, Fig-2). Of the 9 isolates from PAR suspected positive animals, 3 and 6 isolates were identified as capsular type A and D, respectively. On the other hand, all the 6 isolates from apparently healthy animals contained capsular type A only.

Detection of *toxA* gene by *toxA* gene specific PCR assay: All the 15 isolates tested for the *toxA* gene were found to be negative for an expected amplicon of 1230 bp.

Randomly amplified polymorphic DNA - PCR (RAPD-PCR): RAPD-PCR of all the 15 *P. multocida* isolates provided a range of amplicons between 400-1400 bp

with a high degree of discrimination between capsular type A and D. All 9 isolates of capsular type A showed similar banding pattern, and similarly, all 6 isolates of capsular type D exhibited same banding pattern (Fig - 3).

Antimicrobial sensitivity test: PAR in pigs can be treated with potentiated sulphonamides, ampicillin, tetracyclines, ceftiofur or enrofloxacin. Support therapy such as additional food and electrolytes may be necessary. In the present study, majority of the isolates exhibited 100% sensitivity against amoxicillin, cephalexin, chloramphenicol, ciprofloxacin, co-trimoxazole, enrofloxacin, erythromycin, gentamicin, ofloxacin, rifampicin and tetracycline. However, three antibiotics did not show 100% sensitivity. The order of sensitivity were oxytetracycline (93%) followed by ampicillin (87%) and amikacin (47%) (Table-3).

Discussion

The PM-PCR assay used in this study was highly specific and sensitive and direct use of the confluent growth culture provided rapid detection of *P.*

Table-3. Antimicrobial drug sensitivity assay of 15 *P. multocida* isolates

Sr. No.	Antimicrobial agents	Concentration per disc	Sensitive(n= 15)	Resistance(n = 15)
1	Amikacin (AK)	30 mcg	7 (47%)	8 (53%)
2	Amoxicillin (AM)	30 mcg	15 (100%)	-
3	Ampicillin (A)	10 mcg	13 (87%)	2 (13%)
4	Cephalexin (CP)	30 mcg	15 (100%)	-
5	Chloramphenicol (C)	30 mcg	15 (100%)	-
6	Ciprofloxacin (CF)	5 mcg	15 (100%)	-
7	Co-trimoxazole (CO)	25 mcg	15 (100%)	-
8	Enrofloxacin (EX)	10 mcg	15 (100%)	-
9	Erythromycin (E)	15 mcg	15 (100%)	-
10	Gentamicin (G)	10 mcg	15 (100%)	-
11	Ofloxacin (OF)	5 mcg	15 (100%)	-
12	Oxytetracycline (O)	30 mcg	14 (93%)	1 (7%)
13	Rifampicin (R)	5 mcg	15 (100%)	-
14	Tetracycline (T)	30 mcg	15 (100%)	-

multocida, regardless of the purity of the samples. Similar method of bacterial isolation was also followed by other research groups in India and abroad [8, 9]. In routine diagnostic procedure, it is difficult to obtain a pure culture of *P. multocida* from clinical samples because of contaminants and/or death of organisms. Therefore, only PM-PCR positive samples could yield isolation of the organism.

All the 21 PM-PCR positive cultures in BHI broth were inoculated in mice individually for isolation of *P. multocida*. The organism was isolated from heart blood and spleen of dead mice. When the same samples were inoculated directly on 5% sheep blood agar, isolation rate of *P. multocida* was poor (70%). Similar observations were also recorded by other workers with a success rate of approximately 18.0%, because of frequent overgrowth of contaminants [10]. Use of PM-PCR and subsequent mice inoculation for screening of nasal swabs drastically reduced the time for isolation of *P. multocida*.

The present study recorded relatively low recovery of *P. multocida* from nasal swabs collected from Mizoram. This might be due to the random sampling of the animals. Although the sample size might be low for any conclusive remarks, it revalidates the fact that *P. multocida* is a common inhabitant of pig in the nasopharyngeal area. Isolation of *P. multocida* from apparently healthy pigs indicated that these animals were prone to disease under stress conditions. Kalorey *et al.* [11] have also recorded an outbreak of pasteurellosis with high mortality in indigenous pigs in India.

The PCR based capsular typing assay [11] was found to be a rapid and extremely reliable assay for determining the capsular types of a large number of *P. multocida* isolates. In the present study, 9 (60%) and 6 (40%) isolates were capsular type A and D, respectively. None of the isolates were untypeable. Capsular type F and untypeable are uncommon in pigs but are often isolated from avian species. However, Choi *et al.* [12] identified 21 untypeable strains among 203 *P. multocida* isolates from pneumonic pigs in Korea. The present study showed higher percentage of capsular type A (60%), compared to capsular type D (40%), which is in agreement with previous studies [12, 13].

Toxigenic *P. multocida* is recognized as a causative agent of PAR in pigs. It has been isolated with almost equal frequency from pigs with or without the signs of PAR. However, the herds with clinical PAR had a higher prevalence of capsular type D toxigenic *P. multocida* and a lower prevalence of capsular type A non-toxigenic *P. multocida* [8,14]. Moreover, pigs with signs of respiratory tract infection mainly developed the symptoms similar to pneumonia. The result of this study is also in accordance with the previously published reports by various research groups [9]. Ranjan *et al.* [15] also described that *tox A* gene based PCR can be used for direct analysis of toxigenic *P. multocida* without additional hybridization. This assay appears to be the most sensitive and effective method for large scale analysis of nasal and tonsillar swabs.

RAPD-PCR showed that capsular type A and D *P. multocida* isolates exhibited two different banding patterns ranging between 400-1400 bp (Fig - 3). The correlation between RAPD pattern and capsular type helped in differentiating the isolates belonging to the same species. In the present study, it was also observed that RAPD pattern of *P. multocida* capsular type A was different from that of capsular type D, but no discriminatory patterns were recorded within the same capsular type (Fig.-3). Dutta *et al.* [6] observed the polymorphism in 33 isolates of *P. multocida*, isolated from rabbits and reported it as an efficient and reproducible technique. Dutta *et al.* [6,16] characterized *P. multocida* strains of different serotypes by RAPD and observed a unique banding pattern for individual serotypes.

Majority of the isolates were sensitive to amoxicillin, cephalexin, chloramphenicol, ciprofloxacin, co-trimoxazole, enrofloxacin, erythromycin, gentamicin, ofloxacin, rifampicin and tetracycline (100%) followed by oxytetracycline (93%), ampicillin (87%) and amikacin (47%). The lower sensitivity of amikacin could be due to the gradual development of resistance towards these antimicrobial drugs. Higher efficacy of enrofloxacin and ofloxacin along with chloramphenicol has also been reported by Sharma *et al.* [17] in an *in vitro* study. Enrofloxacin and chloramphenicol were found to be quite effective against pasteurellosis by several research groups [18].

Conclusion

Although, no isolates exhibited *tox*A gene by PCR, isolation of *P. multocida* of capsular type A and D may be an indication of their possible role in PAR in pigs of Mizoram. In the last two decades, shift in antibiotic sensitivity spectrum of Pasteurella is well evident from observed clinical resistance against conventional antibiotics, which lead to problems in treating the animals suffering from pasteurellosis. This warrants the need for pre-testing of antibiotics sensitivity against Pasteurella, so as to find out an effective antimicrobial agent to be used by the veterinarian.

Authors' contributions

ZV: Collection of Samples, processing for isolation and identification of *P. multocida*. TKD: Planning of research methodology, technical support, manuscript preparation. PR: Processing of samples for PCR and analysis. JB: Antibiotic sensitivity assay of the isolates. RC: Overall supervision of the work, editing of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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