



# Transmission of Novel Bacterial Pathogens through Pigs Transported from Myanmar to Mizoram

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## ABSTRACT

**Background:** Illegal migration of pigs/piglets from Myanmar to Mizoram is a common practice to meet the local demands. The migrated animals are suspected as potential carrier of various microbial pathogens. The present study was conducted on isolation, identification and molecular characterization of major bacterial pathogens (*Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae* and *Pasteurella multocida*) in pigs illegally migrated from Myanmar to Mizoram.

**Methods:** A total of 209 rectal swabs and 209 nasal swabs were collected from apparently healthy migrated pigs during October 2018 to April, 2019. All the samples were processed for PCR based detection of target bacterial species followed by isolation and identification by bacteriological techniques. The bacterial species were further confirmed by BD Phoenix automated bacterial identification system and selected virulence genes of the bacterial species were determined by specific PCR assay.

**Result:** By species specific PCR, 110 samples were found to be positive for selected bacterial species, of which 20 (9.57%), 1 (0.478%), 86 (41.15%), 2 (0.956%) and 1 (0.478%) were *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis*, *M. hyopneumoniae* and *P. multocida*, respectively. A total of 52 bacterial strains were isolated and identified, of which 11, 1, 39 and 1 were *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis*, *M. hyopneumoniae* and *P. multocida*, respectively. Virulence genes were detected in *A. pleuropneumoniae* and *H. parasuis* isolates. Based upon the published literatures, this is the first ever report of isolation and identification of pathogenic *A. pleuropneumoniae* and *H. parasuis* in pigs in India.

**Key words:** Bacteria, Mizoram, Pigs, Transboundary.

## INTRODUCTION

Animal husbandry is one of the major livelihood sources for the majority of the population in the North-East Region (NER) of India. The pigs occupy the unique position as it is considered as a taboo in the socio-cultural life of tribal people (Das and Bujarbaruah, 2005). In Mizoram, the North-eastern state of India, pig is the most preferable and important livestock and plays a major role in the livelihood of the small farmers. Due to non-availability of healthy piglets through state agencies and multiple disease outbreaks the gap between the supply and demand is wide. The piglets are being transported across the domestic and international borders into Mizoram to meet the excess demands. Due to the uncontrolled migration of young and adult pigs in the state, there is ample chance of introduction of various infections among the pig population of Mizoram and recent outbreaks of porcine reproductive and respiratory syndrome (PRRS) in 2013, 2015 and 2018 are considered as valid evidence (Rajkhowa *et al.*, 2016; Zohlimpuia *et al.*, 2018).

Besides the major viral pathogens, many bacterial agents including *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae* and *Pasteurella multocida* are recognized as major etiological agents of various porcine diseases. *B. bronchiseptica* and *P. multocida* are commensal microflora in the upper respiratory tract of pigs but develop progressive atrophic rhinitis in immunocompromised animals

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(Donko *et al.*, 2003). *H. parasuis* is the causative agent of Glässer's disease of pigs, one of the major economically important diseases in pig industry which shows fibrinous polyserositis, polyarthritis and meningitis (Ferri *et al.*, 2000). *M. hyopneumoniae* is one of the primary pathogens associated with enzootic pneumonia and porcine respiratory disease complex (PRDC) in pigs (Thacker and Minion, 2012). *A. pleuropneumoniae* is an economically important respiratory pathogen associated with acute and often fatal

porcine pleuropneumonia that causes great economic losses to the swine industry.

In India, diseases of pigs associated with various bacterial pathogens including *Bordetella* spp., Mycoplasmas and Pasteurellae are reported earlier by various workers (Kumar *et al.*, 2014; Behera *et al.*, 2018; Dutta *et al.*, 2009) but no report on detection of *A. pleuropneumoniae* and *H. parasuis* are available. In addition, no scientific study has undertaken to record the bacterial pathogens carried by the pigs illegally transported from Myanmar to Mizoram. The present study was undertaken to detect and identify the five major bacterial pathogens (*A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis*, *M. hyopneumoniae* and *P. multocida*) in pigs transported from Myanmar to Mizoram. This is probably the first ever scientific evidence on transboundary transmission of bacterial pathogens through pigs between two countries.

## MATERIALS AND METHODS

### Collection of samples

All the samples were collected from the pigs illegally transported from Myanmar to Champhai district of Mizoram during October 2018 to April, 2019. A total of 209 nasal swabs and 209 rectal swabs were collected under aseptic condition using sterile absorbent cotton swabs (HiMedia, India). All the samples were transported to the laboratory under cold chain for further processing.

### Preparation of samples for screening

All the samples were inoculated in 5 ml sterile Brain Heart Infusion (BHI) broth (HiMedia, India) and incubated at 37°C for 18 hours under constant shaking (120 rpm) in a shaking incubator. After overnight incubation, 1ml of bacterial culture was transferred to a microcentrifuge tube (Eppendorf, Germany) and centrifuged at 8,000 rpm for 10 minutes at 4°C. The cell pellet was washed three times in sterile normal saline solution (0.85%) and finally resuspended in 300 µl of nuclease-free sterile distilled water. The cell suspension was boiled in a water bath (100°C) for 5 minutes followed by snap chilling at -20°C. The cellular debris was sedimented by centrifugation at 8,000 rpm for 5 minutes. The supernatant was used as the template for PCR assay. The left over (4 ml) bacterial culture was stored at 4°C for further use.

### PCR based screening of the bacterial lysates

All the bacterial lysates were screened for detection of target bacterial species (*A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis*, *M. hyopneumoniae* and *P. multocida*) by PCR assay using specific oligonucleotides primers as mentioned in Table 1. PCR was performed in 25 µl reaction volume containing Dream Taq™ Buffer (10X), 2.5 µM of each dNTPs, 20 pM of each specific primers, 1 Unit of Dream Taq™ DNA polymerase and 4.0 µl of bacterial lysates. The reactions were performed with cyclic conditions as shown in Table 2.

### Isolation and identification of target bacteria

All the samples found to be positive for target bacteria (*A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis*, *M. hyopneumoniae* and *P. multocida*) by specific PCR assay were further processed for isolation of bacteria by standard bacteriological technique. After incubation, in each plate, based upon the colony characteristics, minimum 5 pure colonies were selected randomly and subjected to Gram's staining (Quinn *et al.*, 1994). Each selected bacterial colonies were screened for identification up to species level by the BD Phoenix™ automated bacterial identification system as per the guidelines of the manufacturer. All the identified bacterial colonies were further subcultured on specific media for growth and further storage. The bacterial isolates were stored as a pure culture on semisolid agar at 4°C and also as glycerol stock (50% glycerol in LB broth) at -80°C for further use.

### Detection of specific virulence genes in the bacterial isolates PCR assay

All the bacterial isolates were subjected to PCR assay for determination of selected virulence genes using specific oligonucleotide primers (Table 1). Bacterial lysates were prepared as mentioned earlier. PCR was performed in 25 µl reaction volume containing Dream Taq™ Buffer (10X), 2.5 µM of each dNTPs, 20 pM of each specific primers (Table 1), 1 Unit of Dream Taq™ DNA polymerase and 4.0 µl of bacterial lysates. The reactions were performed with cyclic conditions as shown in Table 2.

All the amplified products were analyzed by agarose gel electrophoresis (1.5% agarose in 1XTAE) at 120 V/m for 40 minutes and stained with ethidium bromide (0.5 µg/ml). The products were visualized with a UV trans-illuminator and imaged with a gel documentation system (Alpha Imager, USA). A known molecular weight marker (100 bp DNA ladder) (BR Biochem, India) was also used for each run to compare the amplicon size.

## RESULTS AND DISCUSSION

### Detection of bacterial pathogens by specific PCR assay

By species specific PCR assay, a total of 110 samples were found to be positive for presence of selected bacterial pathogens, of which 20 (9.57%), 1 (0.478%), 86 (41.15%), 2 (0.956%), 1 (0.478%) and 1 (0.478%) were confirmed as *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis*, *M. hyopneumoniae* and *P. multocida*, respectively (Table 3).

### Isolation and identification bacterial pathogens

Of the 110 PCR positive specimens, a total of 52 bacterial strains were isolated and identified by the standard bacteriological techniques and BD Phoenix™ automated bacterial identification system. A total of 11, 1, 39 and 1 bacterial isolates were identified as *A. pleuropneumoniae*, *B. bronchiseptica*, *H. parasuis* and *P. multocida*, respectively. *M. hyopneumoniae* could not be isolated (Table 3).

**Detection of specific virulence genes in the bacterial isolates PCR assay**

A total of 6 *A. pleuropneumoniae* isolates were recorded as positive for *ApxIA* and *ApxII* genes. In addition, 12 *H. parasuis* isolates were also recorded as positive for *vtaA* and *capD* genes. None of the other bacterial isolates were found to be positive for any other selected virulence genes.

Although pig husbandry is one of the major backbones of the rural economy of Mizoram, it used to suffer from several setbacks due to various diseases contributing a big hindering factor towards the steady growth of pig husbandry.

On many occasions, the piglets are being transported across the domestic and international borders due to shortage in supply of good quality piglets to the farmers for rearing. The present study was undertaken to find out the occurrence of porcine bacterial pathogens in the pigs migrated from Myanmar to the adjoining district Champhai in Mizoram.

*H. parasuis* was recovered in the highest number followed by *A. pleuropneumoniae*. Both the bacteria are considered to be the most commonly detectable normal flora of pig respiratory tract, which may develop clinical infestations under stress, particularly in immunocompromised conditions

**Table 1:** List of oligonucleotide primers used in the present study.

Name of bacteria	Primer name	Primer sequence (5'-3')	Expected amplicon size (bp)	References
<b>Primers used for species specific detection of target bacteria</b>				
<i>A. pleuropneumoniae</i>	APXIVA-1	F-TGGCACTGACGGTGATGA R-GGCCATCGACTCAACCAT	422	Schaller <i>et al.</i> (2001)
<i>B. bronchiseptica</i>	Fla2 Fla4	F-AGGCTCCCAAGAGAGAAAGGCTT R-TGGCGCCTGCCCTATC	237	Hozbor <i>et al.</i> (1999)
<i>H. parasuis</i>	16S	F-AGAGTTTGATCATGGCTCAGA R-AGTCATGAATCATACCGTGGTA	1,391-1,394	Olvera <i>et al.</i> (2006)
<i>M. hyopneumoniae</i>	16S	F-GAGCCTTCAAGCTTACCAAGA R-TGTGTTAGTGACTTTTGCCACC	649	Kurth <i>et al.</i> (2002)
<i>P. multocida</i>	KMT1T7 KMT1SP6	F-ATCCGCTATTTACCCAGTGG R-GCTGTAAACGAACTCGCCAC	460	Townsend <i>et al.</i> (1998)
<b>Primers used for determination of virulence genes of bacterial isolates</b>				
<i>A. pleuropneumoniae</i>	apxIA apxIB apxII	F-ATCGAAGTACATCGCTCGGA R-CGCTAAGCTACGACCGAAC F-TTATCGCACTACCGGCACTT R-TGCAGTCACCGATTCCACTA F-GAAGTATGGCGAGAAGAACG R-CGTAACACCAGCAACGATTA	723 811 963	Rossi <i>et al.</i> (2013)
<i>B. bronchiseptica</i>	bvgA cyaA flaA	F-AATTTGCGAGCCATTCTTTGAC R-GATCAGACTGCGGGGTACAG F-GGTGCGAATCCGTTCAATCGACTA R-TTCCAGTACATCCGGCGAGGACTTC F-CGCCGCCAACCAGTC R-GTACGTACTGCCATGGCCCCG	768 1185 736	Kumar <i>et al.</i> (2014)
<i>H. parasuis</i>	vtaA hhdBA fhuA lsgB capD	F-TTTAGGTAAAGATAAGCAAGGAAATCC R-CCACACAAAACCTACCCCTCCTCC F-ATCTTGCCCTGATTAGAGAGTAGGAGT R-GTGAATATAGCCCTTATCCAAATAGGC F-ATGGTTTGGTTGTAATGGAGTATC R-AACAACGCCAGCTAGGCTTGACT F-ATGAATTTGATTATTTGTATGACTCCATTT R-CTATTGGCATGTGTAGTCAATTACTTC F-CGAAGGGAGTGTTTCTATCA R-GAGTTTCTCACCAGGTCTAA	460 557 563 942 958	Lawrence and Bey (2015)
<i>P. multocida</i>	ompH ompA toxA	F-CTGGTTTAGCGCTTGGTGTT R-TCTACCCCAAGCTGCTTCAA F-AGCGCGTAGATTACAGACCA R-GTGACCTGTTGCGCTGATAG F-GGTAAAGAGTTTGCCGTGGA R-CGAGGCTTTGTGAAAAGAGG	242 350 200	Rajkhowa <i>et al.</i> , (2012)

**Table 2:** Thermal cycling conditions for the detection of various specific bacterial genes in the present study.

Name of the target genes	Initial denaturation	Denaturation	Annealing	Elongation	Final extension	Number of cycles
APXIVA-1	94C; 5 min	94C; 30 sec	53C; 30 sec	72C; 30 sec	72C; 4 min	35
Fla2 and Fla4	94C; 5 min	94C; 1 min	60C; 50 sec	72C; 1 min	72C; 4 min	35
<i>H. parasuis</i> (16S)	94C; 5 min	94C; 1 min	55C; 15 sec	72C; 20 sec	72C; 4 min	30
<i>M. hyopneumoniae</i> (16S)	94C; 5 min	94C; 30 sec	60C; 45 sec	72C; 30 sec	72C; 4 min	35
KMT1T7 and KMT1SP6	94C; 5 min	94C; 45 sec	56C; 45 sec	72C; 45 sec	72C; 4 min	30
apxIA, apxIB and apxII	94C; 5 min	94C; 30 sec	60C; 30 sec	72C; 45 sec	72C; 4 min	35
bvgA, cyaA and flaA	94C; 5 min	94C; 50 sec	57C; 60 sec	72C; 90 sec	72C; 4 min	35
vtaA	94C; 5 min	94C; 45 sec	64C; 45 sec	72C; 60 sec	72C; 4 min	25
hhdBA, fhuA	94C; 3 min	94C; 30 sec	55C; 60 sec	72C; 90 sec	72C; 4 min	32
ompH, ompA and toxA	94C; 5 min	94C; 30 sec	56C; 45 sec	72C; 45 sec	72C; 4 min	30
lsgB, capD	94C; 5 min	94C; 45 sec	56C; 45 sec	72C; 90 sec	72C; 4 min	30

**Table 3:** Details of PCR based detection and bacteriological isolation and identification of various bacterial species in the specimens collected from pigs transported from Myanmar to Champhai district of Mizoram.

Bacterial species	Number of samples positive for specific PCR	Number of bacteria isolated and identified	Number of bacterial isolated posses virulence gene (s)
<i>Actinobacillus pleuropneumoniae</i>	20	11	6
<i>Bordetella bronchiseptica</i>	1	1	-
<i>Haemophilus parasuis</i>	86	39	12
<i>Mycoplasma hyopneumoniae</i>	2	0	-
<i>Pasteurella multocida</i>	1	1	-
Total	110	52	

(Galofré-Milà *et al.*, 2017). The rate of isolation and identification of bacterial pathogens from any kind of hosts are dependent on various factors: sampling pattern, season, time of collection, treatment with antibacterial drugs, processing in the laboratories, media used, methods of identification and confirmation and so on. In an extensive epidemiological study conducted between 2013 and 2017, a total of 19673 bacterial strains were isolated from 44175 samples collected from 9661 pig farms that distributed in 16 Chinese major pig breeding provinces, where *Streptococcus suis*, *H. parasuis*, *E. coli*, *P. multocida*, *A. pleuropneumoniae*, *B. bronchiseptica*, *S. enterica* and *Erysipelothrix rhusiopathiae* were isolated at the rate of 16.9%, 9.7%, 6.3%, 3.4%, 0.3%, 1.5%, 2.3% and 0.9%, respectively. Detection of *P. multocida*, *B. bronchiseptica*, *M. hyopneumoniae* and *Salmonella enterica* were widely variable depending upon the geographical location and husbandry conditions (Zhang *et al.*, 2019). In an earlier study, Varte *et al.* (2014) reported 3.15% of pig population of Mizoram as carrier of *P. multocida* of serogroup A and D, although none of them were toxigenic strains and associated with progressive atrophic rhinitis. *P. multocida* and *B. bronchiseptica* are usually surviving simultaneously in the upper respiratory tract of pigs and associated with progressive atrophic rhinitis. Therefore, the detection of both the pathogens from the pigs of this region indicates the carrier status and may aggravate the condition with progressive atrophic rhinitis under favourable conditions.

The prevalence of *H. parasuis* in the nares has been linked to increased risk of infection by other pathogens. In contrary, Vahle *et al.*, (1997) reported that detection of *H. parasuis* from the nasal cavity and tonsils do not provide a conclusive diagnosis for Glässer's disease in the animals, as the organisms are considered a commensal in the upper respiratory tract and both pathogenic and non-pathogenic strains can be isolated from these sites. Thus, *H. parasuis* present in nasal cavity or tonsils might not necessarily be the causal serovar for the disease (Olvera, 2006) and sampling the internal organs is recommended to determine the disease-causing serovar of *H. parasuis*. But the determination of such virulent organism from the healthy animals is always an indication of possible infection under immunocompromised conditions. So far, no published literatures could be traced on *H. parasuis* infestation in pigs in India, hence the present data could not be compared. In China, the incidence of *H. parasuis* in healthy and infected pigs are reported (Zhang *et al.*, 2019), where *H. parasuis* isolation was more successful in weaner pigs (22.6%, 192/849), followed by finisher pigs (9.3%, 43/463) and sows (2.5%, 9/363).

*A. pleuropneumoniae*, the causative agent of porcine contagious pleuropneumonia is worldwide in distribution. Acute disease, characterized by fibrino-hemorrhagic and necrotizing pleuropneumonia, is often fatal. Asymptomatic carriers of the bacterium, either those having survived acute disease or those that were subclinically infected, may harbor



the organisms in nasal cavities, tonsillar crypts and chronic lung lesions, thus becoming a source of infection for naïve subpopulations (Klinkenberg *et al.*, 2014). The estimation of the economic burden of this disease is mainly based on the occurrence of acute outbreaks characterized by high mortality, loss in production and high medical costs. Results from different studies about the impact of *A. pleuropneumoniae* infections on production parameters vary greatly. Few studies have confirmed the broadly accepted perception that average daily weight gain and feed conversion rates are negatively affected by the disease, mainly in pigs with chronic lung alterations (Hoflack *et al.*, 2001). A reduction in daily weight gain by 33.6% and a decrease in feed efficiency ranging from 0.77% to 25.5% have been reported (Holmgren *et al.*, 1999). The organism is reported from pigs of most of the countries (Sassu *et al.*, 2018) but so far, no published data available from India. As there is no published data from India is available, we could not compare our data. This is probably the first report on the isolation and identification of *A. pleuropneumoniae* and *H. parasuis* from Indian pigs.

It is established that pigs illegally migrated from Myanmar to Mizoram are carrying various bacterial pathogens. In the present study, no attempt has been made to use any genotypic tool for comparison of genetic variations between the pathogens isolated from the native pigs of India and pigs migrated from Myanmar. The Mizoram state of India shares a porous international border of 510 km with Myanmar. Hundreds of pigs regularly enter into Mizoram through the international border illegally, not only for slaughtering but also for the breeding purpose (Rajkhwa *et al.*, 2016). Two important bacterial pathogens, *H. parasuis* and *A. pleuropneumoniae* are not reported from any pig producing farm of India so far. Therefore, this may be considered as the first-ever report on isolation and identification of *Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* in pigs in India and interestingly, both the organisms were isolated from pigs migrated from Myanmar to Mizoram.

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## Conflict of interest

The authors certify that there is no conflict of interest in publication of the article.

## Ethical statement

Samples were collected from the clinically infected animals without any invasive techniques with the due permission of Institutional Animal Ethics Committee, CVSc and AH, CAU, Aizawl, Mizoram.

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