Summary
SUMMARY

The present study was undertaken for examining the causative agents and molecular characterization of the organisms with special emphasis on *B. bronchiseptica* involved in the atrophic rhinitis in pigs. During the study period the respiratory diseases especially atrophic rhinitis and bronchopneumonia in pigs were observed in different seasons of the year. The disease was recorded in maximum numbers during winter months, with reoccurrence during the start of rainy season in the months of April and May, when the weather and environmental conditions were drastically changed.

In the present study, 47 *B. bronchiseptica* and 5 *P. multocida* were recovered from the atrophic rhinitis and bronchopneumonia cases from fourteen different outbreaks. Based on the results, *B. bronchiseptica* was found to be the primary etiological agent for the atrophic rhinitis and bronchopneumonia in pigs. Bordet Gengou agar supplemented with 10% sheep blood was found as the most successful solid agar medium for isolation of *B. bronchiseptica*. On simple blood agar media, *B. bronchiseptica* colonies were white, dome shaped and hemolytic. β-hemolysis was observed maximum on Bordet Gengou agar supplemented with 10% sheep blood. *P. multocida* were recovered especially from nutrient agar supplemented with 10% sheep blood. Ultrastructural study of *B. bronchiseptica* by scanning electron microscopy showed the distribution pattern of bacteria in a single bacterial colony.

In the colonization study, heavy mucus deposition was seen in the tracheal surface along with necrosis. The lungs showed necrotic lesion and foci on the entire lung surface. With scanning electron microscopy, the rupture
of the membrane was observed over the entire tracheal epithelia. Scanning
electron microscopy revealed both small and large numbers of *B. bronchiseptica*
cells became entangled with the cilia of the tracheal epithelial cells. The tracheal
cilia lost their normal architectural arrangement in some area, and the
openings increased on the top of the Goblet cells along with broken tips. Severe
necrosis in tracheal cell wall with the presence of fibrilar and globular
appearance of mucus were also observed in tracheal layer. In the affected lungs,
abnormally high red blood cell’s population was observed with the deposition
of cellular debris. Deformed alveolar cells with colonization of bacteria were
also observed in the lung surface. The use of rabbit as experimental animal
provides a good means to study host-pathogen interaction for *B. bronchiseptica*.

The Congo red binding test and urease activity of *B. bronchiseptica*
revealed the isolates were in Bvg* (virulent) phase. All the *B. bronchiseptica*
isolates were strongly positive for hemagglutination reaction with sheep, rabbit
and pig RBCs while the isolates failed to elucidate any hemagglutination
reaction with RBCs from other animal species of cattle, goat and duck. *B.ronchiseptica* isolates produced strong hemolysis only with Sheep blood and
weak hemolysis with goat, cattle and rabbit blood but failed to produce any
hemolysis with chicken RBCs. In the phase modulation study, incubation of
solid media plates at room temperature (24°C) clearly gave the distinguishing
Bvg phase characteristics. The two major PVs could be divided as: domed,
smooth colonial surface, and hemolytic (Dom+ Scs+ Hly+) in the non modulated
(Bvg+) phase and flat, smooth colonial surface, and non hemolytic (Dom- Scs+
Hly) in the modulated (Bvg') phase. The difference between the normal phase
(Bvg+) and the modulated phase (Bvg') were clearly observed in the polypeptides
analyzed by SDS-PAGE.
Six *B. bronchiseptica* isolates produced severe necrotic lesion in the dermonecrotic test in rabbit followed by moderate necrotic lesion in six isolates and weak dermonecrotic lesion in six isolates. However 2 isolates did not produce any dermonecrotic reaction. The toxin treated vero cells rapidly underwent morphological changes within one day as observed by inverted microscope. Vero cell monolayer developed foci or patches of dense cell clusters surrounded by enlarged cells with many polynucleated cells.

In the PCR analysis all the 47 *B. bronchiseptica* isolates showed the presence of major toxin genes. All the *B. bronchiseptica* isolates produced an amplicon sizes of 600 bp, 599bp, 745 bp, 320 bp, 1767 bp, 301 bp, 454 bp and 598 bp respectively, partially representing *bvgAS, dnt, achlyA, fhaB, fhaC, fim2, fim3* and *tcfA* genes. The polymerase chain reaction assay described in the present study may prove to an improvement of the present methods for surveillance of bordetellosis and may provide a more accurate means for the diagnosis of *B. bronchiseptica*. 3mM iron concentration was found optimum for the *in-vitro* toxin production among the isolates. With the increase in iron concentration beyond 3mM, the toxin production did not show any consistent result.

The dermonecrotic toxin was purified by fast protein liquid chromatography (FPLC) system. The homogeneity of purified DNT was checked by SDS-PAGE analysis. A sharp single band was detected in the SDS-PAGE analysis with molecular weight of 160 kDa (approx). The immunogenecity of the purified DNT preparation was also detected by western blot analysis, which gave a band of approximately 160 kDa. The homogenous *B. bronchiseptica* DNT preparation and the resulting *B. bronchiseptica* DNT antiserum available in the present study would be useful tools to clarify the
hypothesis that *B. bronchiseptica* DNT is responsible for the pathogenesis of swine atrophic rhinitis along with in the preparation of diagnostic antigen against Bordetellosis.

The polypeptide profiles of *B. bronchiseptica* isolates were carried out in a continuous 5-15% gradient polyacrylamide gel. A total of 18-20 numbers of polypeptides were observed including seven major bands of high intensity and thirteen minor bands of low intensity in the molecular weight range of 20 to 100 kDa. In the lipopolysaccharide study all the isolates showed the presence of LPS bands. Two major forms of LPS bands (band A and band B) were detected in all the fourteen *B. bronchiseptica* isolates. The polypeptides and LPS profiles suggest the highly clonal nature of *B. bronchiseptica* isolates associated with the atrophic rhinitis and bronchopneumonia in swine irrespective of the disease condition, geographical location and place of isolation. However, LPS profiles of large number isolates can also answer the question of whether LPS profiles are useful as an additional approach for differentiating *B. bronchiseptica* isolates.

In the random amplified polymorphic DNA (RAPD) analysis, the random primer OPG-2 generated ten different fingerprint profiles while OPG-5 generated twelve different fingerprint profiles of 47 *B. bronchiseptica* isolates analyzed. The results of this study establish that when an appropriately chosen set of primers is employed, RAPD analysis provides an alternative rapid, reproducible and powerful genomic typing method for *B. bronchiseptica*.

Alkali-lysis extraction method revealed the presence of plasmids among *B. bronchiseptica* isolates. The isolates were having either single or double plasmids in the molecular range of 25-26 and 14-16 kb respectively. Out of forty seven, twelve (25.53%) of the *B. bronchiseptica* isolates harbored single
plasmid whereas thirty five (74.47%) isolates harbored double plasmids. Overlaid graph arising from the dendogram analysis of plasmids showed the similarity range from 67.20-94.49% among the isolates. In the cluster analysis by Un-weighted pair group method using arithmetic averages (UPGMA) using Pearson product at 4% autofit Cluster I, cluster II, cluster III and cluster IV isolates showed similarity of 93.99%, 88.70-99.33%, 98.87-99.03% and 91.57-96.48% respectively among them. In the present study, no marked differences were observed among the isolates of different geographical location and year of isolation in plasmid DNA distribution pattern. Very high percentage of isolates carrying 2 plasmids from Meghalaya in comparison to Assam could be incidental and no satisfactory explanation appears to be possible.

In the REA analysis, DdeI and Hinfl were found to be the best restriction enzymes in generating comparable fingerprints. Both the restriction endonucleases resulted in well-separated and distinct bands. DdeI restriction enzyme generated 32 numbers of bands in molecular range of 1.5-12.7 kb, while Hinfl generated 30 numbers of bands in 1.2-11.0 kb molecular range. Overlaid graph arising from the dendogram analysis of REA profiles showed the similarity range among isolates from 94.97-99.25%. In the cluster analysis by Un-weighted pair group method using arithmetic averages (UPGMA) using Pearson product at 4% autofit, cluster I, cluster II, cluster III and cluster IV isolates showed similarity of 99.24%, 98.32-99.62%, 99.04-99.70 and 99.31-99.68% respectively among them. The REA results suggest that B. bronchiseptica involved in AR and bronchopneumonia arised from the lineage of a single clone irrespective of geographical locations of isolation and age of the animals. DdeI and Hinfl might be used as restriction enzyme of choice in analysis of markers in epidemiological studies of atrophic rhinitis and
bronchopneumonia involving *B. bronchiseptica* isolates.

Sequence analysis of partial *dnt* gene revealed *B. bronchiseptica* isolates to have 531-555 nucleotides with an open reading frame coding for 177-185 amino acids long DNT protein in the 5' end. *B. bronchiseptica* isolates of present study showed 100% sequence homology with the reference sequences of different geographical origin. However, field isolate Bb25 bearing showed three nucleotide replacements (C_{259} \rightarrow T, A_{472} \rightarrow G, C_{489} \rightarrow A) and Bb41 showed only one nucleotide replacement (T_{481} \rightarrow G). The field isolates Bb14, Bb32, Bb13, Bb43 and reference sequences U59687 and AB020025 showed one nucleotide replacement at position (C_{259} \rightarrow T). The nucleotide sequence analysis also revealed that the *Bordetella* genome is G + C content biased.

Phylogenetic analysis of nucleotide sequence of partial *dnt* gene grouped various isolates including Indian field isolates and reference strains into four major clusters in one lineage. The field isolates Bb27, Bb39, Bb46, Bb22, Bb2, Bb1, Bb20, Bb5 and Bb9 of cluster I showed 100% sequence homology with reference isolates bearing acc. No. BX640434, BX640421, U10527 and BX640449. However, Cluster I is 0.2%, 0.6% and 0.2% divergent from clusters II, III and IV respectively. Cluster II field isolates Bb14, Bb32, Bb13 and Bb43 shared 100% sequence homology with reference sequence U59687 and AB020025 reference strains. However, cluster II was 0.4% divergent from both cluster III and IV. The maximum divergence of 0.8% was found between the isolate Bb41 of cluster IV and isolate Bb25 of cluster III, followed by overall percent divergence among *B. bronchiseptica* field isolates was 0.0 – 0.8%. From the phylogenetic clustering it was noticed that the reference strains of *Bordetella* genus shared the sequence homology with the present isolates irrespective of the geographical region and host origin. Alignment of deduced
amino acid sequences showed that the Indian field isolates Bb25 and Bb41 had one amino acid substitution at position (S\textsubscript{158} $\rightarrow$ G) and (L\textsubscript{161} $\rightarrow$ V) respectively compared to the reference strains. Even though, the Indian field isolates Bb13, Bb25, Bb32, Bb14, Bb43 and reference sequences AB020025 and U59687 showed the nucleotide replacements at position (C\textsubscript{259} $\rightarrow$ T) but, their corresponding amino acids did not show any change.

The nucleotide sequence analysis and its phylogenies suggest close homology (>99%) with 0.0-0.8% divergence among the isolates in the present study. This shows the highly clonal nature of the Indian field isolates recovered from the bronchopneumonia and atrophic rhinitis cases in pigs. This is possible because of unrestricted movement of animals within the region due to sale and purchase of animals, regular shifting of animals from one herd to another and migration of animals from various places to the slaughter houses etc.

Eventhough, the present findings is based on the partial dnt gene sequence, the findings have not only amply established the identity of the Bordetella sp. but also have given an insight into diversions in this limited sequence of dnt gene, which is highly otherwise conserved in the genus level. However, further analysis of full length dnt gene and possibly along with other toxin genes including bvgAS (the virulence control system) will give better understanding of genetic diversity of B. bronchiseptica based on nucleotide sequencing, so that a meaningful conclusion on pathogenic potential of the isolates, as well as host dependent gene expression aspect becomes clear, before really a potential vaccine candidate can be selected. In addition, acquisition of sequence from PCR amplified products and their phylogenetic analysis provides a rapid, accurate and powerful tool for the improvement of genetic analysis and epidemiological investigation.
In the Western blot analysis, 4 immunogenic bands were detected in laboratory adapted isolates in molecular range of approximately 60kDa, 68 kDa, 160kDa and 200kDa respectively. One extra immunogenic band of 40 kDa along with the other four was observed in the wild type isolates. However, the modulated B. bronchiseptica isolates produced only two dominant immunogenic bands of approximate molecular weight 40 and 72 kDa respectively. The cross reactivity of the said polypeptides should be tested with the related bacterial antigens to determine whether those polypeptides have got the potentiality of species specific diagnosis. Further, the change in the modulated 72 kDa protein with molecular weight of 68 kDa of the wild type also needs to be elucidated clearly before the modulated isolates can be considered as vaccine candidate.

Cloning of B. bronchiseptica dnt and achlyA genes by using E. coli DH5 alpha strain showed successful production of recombinants in blue white screening assay. Further detection of both toxin genes by polymerase chain reaction also revealed the presence of specific band of target toxin genes, thus confirming the successful cloning of dnt and achlyA genes.

Eventhough, the findings reported in the present study is based on the isolation of B. bronchiseptica and P. multocida and molecular characterization of B. bronchiseptica from Meghalaya and Assam states, further systematic studies from other pig producing states in the North Eastern region will give actual picture of causative agents for atrophic rhinitis and bronchopneumonia in pigs before taking further step towards controlling the disease and developing a cost effective vaccine.