5. Discussion.

The primary aim of this study was to find out the incidence rate and genotype distributions of PCV-2 and PPV of the porcine population of Northeastern India, with special emphasis on Meghalaya. Infection of PCV-2 in a farm often represents subclinical infection (Ladekjær-Mikkelsen *et al.,* 2002), in which pigs may act as potential viral shedders (Brunborg *et al.,* 2004). Therefore, it is necessary to observe PCV-2 infection in a farm either serologically or by PCR to prevent the pigs from PWMS and minimize economic losses. The overall sero-prevalence rate of PCV-2 and PPV in this region were 67.84% and 37.63%, respectively; and 27.51% pigs represented concurrent infection of both the viruses. As compared to the PCV-2 sero-prevalence in China of 10-90% (Ge *et al*, 2011) and in Slovakia (54%) (Csank *et al.*, 2011), the prevalence of 67.84% in this region is quite high. When sero-prevalence was compared state-wise, highest PCV-2 sero-prevalence was found in Manipur (98%), followed by Nagaland (78.33%), Meghalaya (78.24%), Mizoram (70%), Arunachal (61.36%) and Assam (38.96%); whereas, Nagaland with sero-prevalence rate of (85.83%) was highest for PPV which was followed by Mizoram (66.25%), Meghalaya (37.11%), Manipur (30.07%) and Assam (3%). Trends of PCV-2 Ab titre values' in most of the districts of different states were moderate (intermediate) to high.

Overall detection rate of PCV-2 by PCR in Meghalaya and rest of the Northeast was 25.6% and 10.65%, respectively, which gave an idea about the chances and viability of PMWS in pig population of the region. Castro *et al* (2012), while investigating/studying PCV-2 in 168 aborted foetuses by PCR, found 10.7% of the

samples to be positive for PCV-2. In the same study, they found co-infections with PPV in 7.2% of the investigated foetuses and only 3.6% were found to be mono-infections. Several other studies have demonstrated that PCV-2 is the primary and essential etiological agent in the pathogenesis of PCVAD but experimentally, PCVAD are reproduced most efficiently and consistently when PCV-2 inoculation was done in conjunction with other swine pathogens like PPV (Opriessnig and Halbur, 2012). When infected with PCV-2 many co-pathogens can enhance or induce PCVAD lesions and symptoms and contribute to triggering clinical disease (Rose *et al*, 2011). In one of such studies in Canada, PPV and PCV-2 co-infections were demonstrated in 17.4% (12/69) of pigs with naturally acquired PCVAD (Ellis *et al.*, 2000). However, there are no records of earlier studies on the prevalence of PCV-2 and PPV in this region. In the present study, it was observed that the overall sero-coinfection rate in Meghalaya was 24.68% and 27.51% in the entire Northeast region, which is reasonably higher than reports in Canada and other European countries. Recent reports from China indicated PCV-2 and PPV co-infection rate to be 60% (Sun *et al.*, 2015). Genomic detection of both the viruses at a rate of 1.25% was recorded in our study this was coupled with a high antibody titer, indicating the co circulation of virus antibody complexes. This definitely might add a new dimension to the disease dynamics of PCV-2 and PPV in porcine in India from the field epidemiology point of view.

For the majority of tribal population in the northeast, there is a growing demand for pork due to increasing per capita income, urbanization and changes in lifestyle and food habits. Much of this demand is met from imports from other states and from Myanmar (Suri, 2012) but most of these trading is clandestine, without any disease screening. Porous border between Myanmar and North East India and less

intrastate vigilance on animal's movement were thought to be the main cause of PPRS outbreak in Mizoram during 2013 (OIE 2013). This may provide a new perspective on the epidemiology and status of PCV-2 and PPV among the pig population of the northeastern region.

To date, several genotyping studies on PCV-2 have been reported (Carman *et al.,* 2008; de Boisseson *et al.,* 2004; Grau-Roma *et al.,* 2008). In order to minimize current scientific confusion on genotype names, the EU consortium on porcine circovirus diseases (www. pcvd. org) proposed a unified nomenclature for PCV-2 genotype. The consortium previously proposes naming the three PCV-2 genotypes as PCV-2a, PCV-2b and PCV-2c (Segales *et al.*, 2008). Using this methodology, ORF2 and whole genome sequences of PCV-2 are assigned to different genotypes when the genetic distance between them is at least 0.035 and 0.02, respectively (Segales *et al.,* 2008; Cortey *et al.,* 2011). Recently, consortium considered a fourth one, initially thought to be restricted in China, but nowadays detected worldwide, has been designated as PCV-2d (Guo *et al.,* 2010).

Most of the isolates of PCV-2 in this study were classified as genotype PCV-2b based on ORF-2 sequences. A total of 29 isolates of this study were in 2b genogroup including 11 isolates of Meghalaya. In addition, only 3 isolates from Arunachal were in PCV-2a genogroup and 1 isolate from Manipur was in 2d genogroup. A total of 15 isolates on the basis of ORF-2 sequences were seen to be divergent and represented a variant genotype. The isolates under the variant group were isolated from Meghalaya, Mizoram, Assam and Nagaland.

The genetic distance (ORF-2) of the emergent genotype of this study with respect to other genotypes was 0.08 (PCV-2a), 0.062 (PCV-2b), 0.060 (PCV-2c),

respectively, and formed a distinctly separate branch in the phylogenetic tree. In the year 2009, Wang and his co-workers proposed two new PCV-2 genotypes (PCV-2d and PCV-2e), isolated from Chinese pig. However, the definition of these two new PCV-2 genotypes was consequently found to be wrong (Cortey *et al.*, 2011a), because the definition was solely based on the distance among the complete genomes using the threshold calibrated for the cap gene and no PASC analysis was performed to adapt the threshold calibrated for the cap gene to the complete genomes. PASC analysis with the variant (emergent) genotypes of this study represents very low level of blast alignment (55% to 57%) as well as global alignment (36% to 39%) identity to existing sequences of Genbank. Whereas, blast and global alignment carried out among the the new genotypes represent 98-96% identity. Hence this further strengthens the unique features of the variant group of PCV-2 identified.

PPV is distinguishable from parvoviruses of all other species; it is antigenically related to other parvoviruses such as the canine parvovirus (CPV) and the feline panleukopenia virus (FPV) (Mengeling *et al.*, 1988). It is nowadays accepted that only one serotype of PPV does exist (Mengeling, 2006). Maximum likelihood phylogenetic analysis represent that all the PPV isolates of this study was in group I cluster along with highly virulent strain. Previous experimental study of pregnant sows indicated that at least a German PPV isolate (PPV-27a) of this reference group displayed low homologous neutralizing antibody titre values compared to PPV vaccine and selected field strains, suggesting a possible new antigenic variant of PPV (Zeeuw *et al.*, 2007). All the isolates of this study were very close to each other. This is the first report of a German-like PPV from field samples in India. This provides new molecular data on PPV strains of India, which would be useful for future etiological and epidemiological studies. Concurrent infection with

highly virulent PPV strain may help to increase PCV-2 infection through proliferation of histiocytes and macrophages, cell types in which load of PCV-2 is found higher (Sorden, 2000).

The first report of PCVAD in Indian was in the year of 2014 (Karrupan *et al.,* 2014) but presence of PCV-2 was detected in pig, suffering from reproductive failures in 2010 (Sharma and SaiKumar, 2010). There have been several retrospective studies in other countries that highlighted the presence of PCV-2 in pigs prior to the disease outbreak (Mori *et al.,* 2000, Grierson *et al.,* 2004, Jacobsen *et al.,* 2009). Although all of the PCV-2 genotypes were found in pigs with PMWS, several studies found that isolates from animals that had PMWS often contained PCV-2b, while isolates from animals that did not have the disease contained PCV-2a (An *et al.,* 2007; Grau-Roma *et al.,* 2008). However, it is now established that not all pigs infected with PCV-2 will develop PMWS. More pathogenic viral variants are sometimes able to cause clinical disease in a large number of animals and hence they were isolated more in numbers leading to an increased frequency of PCV-2b sequences in the GenBank database. A genetic switch over from PCV-2a to PCV-2b occurred around 2003 (Dupont *et al.,* 2008; Firth *et al.,* 2009, Cortey *et al*., 2011b). Phylogenetic analysis revealed that PCV-2b genotype was more prevalent in comparison to PCV-2a and PCV-2d as per analysis of uploaded sequences even prior to 2003. Previously reported PCV-2 isolates from Southern part of India belonged to PCV-2b (KF417532.1) whereas isolates from Northern parts of India belonged to PCV-2a (KJ729073.1) and PCV-2b (GU808525.1) genotypes (Karuppannan *et al.,* 2014, Anoopraj *et al.,* 2015). This could indicate variations in the erstwhile circulating strains of the virus. In our study, PCV-2d isolates from Manipur was the first report from this region. Many factors related to the transmission, pathogenesis of PCV-2 that would contributed to its rapid

spread within the porcine population worldwide, the marketing of sub- clinically infected animals, and the selection of these animals for breeding programs might also increase the chances of PCV-2 transmission through semen from sows to piglets around the world (Schmoll *et al.,* 2008).

Capsid protein of PCV-2 is involved in the formation of the homopolymer capsid and is likely involved in translocating the viral genome into the nucleus during virus replication (Nawagitgul *et al.,* 2000). Capsid protein contains four antibody recognition domains; defined as A: 51 to 84; B: 113 to 132; C: 169 to 207 and D: 228 to 233 and alteration within these domains may play crucial role in PCV-2 pathogenesis (Khayat *et al.,* 2011). The deduced amino acid capsid protein sequences of PCV-2 isolates of this study indicated several amino acid substitutions within this domain compared to prototype sequences of PCV-2. Amino acid alignment of these isolates represent, substitution at position 59 (arginine →alanine). Naturally, PCV-2b genotype bears arginine and PCV-2a genotype bears alanine at 59 position of capsid gene. Previously, a virus neutralization study by Huang *et al.,* (2011), using monoclonal Ab (mAb) 8E4 was done and the results showed that a single residue change in Capsid Protein, alanine (found in PCV-2a) to arginine (found in PCV-2b) at position 59, eliminated virus neutralization activity. However, the substitution of an alanine for arginine in PCV-2b failed to restore neutralization of the same and they concluded that presence of alanine at position 59 is necessary for virus neutralization but not solely responsible (Huang *et al.,* 2011). All the variant isolates of this study was seen to possess a unique amino acid substitution i.e. T/Thr at 63 position on capsid genes, compared to reference sequence of PCV-2.

Positions 70-Asp, 71-Met, 77-Asn and 78-Asp were identified as key residues within epitope A. Isolates ML-10 and ML-6 bearing an amino acid substitution at

position 70 i.e., D/Asp to V/Val and G/Gly respectively. A lysine extension present at 234 position of capsid protein may change virus pathogenesis. In recent years, the lysine extension at the C-terminus of the capsid protein have been reported in several countries, including Germany (Knell *et al.,* 2005), Indonesia (Manokaran *et al.,* 2008), China (Wang *et al.,* 2009; Guo *et al.,* 2011a) and Serbia (Savic *et al.,* 2011). PCV-2 with a lysine residue at 234 position in capsid protein showed more virulence in vivo, compared with classical PCV-2a and PCV-2b strain (Guo *et al.,* 2012).

Amino acid comparisons between PPV IDT stain and isolates of this study represents three amino acid substitution i.e., His to Glu at 383 position, Ala to Ser at 414 position and Pro to Thr at 438 position. Some studies have reported that the tropism, host range and haemcoagulation properties of PPV were mainly determined by few changes in the VP1/VP2 genes, which are involved in the expression of structural proteins, such as the viral capsid (Bergeron *et al.,* 1996; Simpson *et al.,* 2002; Zimmermann *et al.,* 2006). Although PPV is preventable through inactivated and attenuated vaccines, it has a high nucleotide substitution rate. But changes in VP-2 capsid protein lead to changes in virus pathogenicity as well as implies to designing new vaccines to prevent new PPV epidemics ((Zeeuw *et al.,* 2007).

Antigenic variations among the PCV-2 strains have been well documented (Lefebvre *et al.,* 2008 a; Shang *et al.,* 2009; Saha *et al.,* 2012). In this study, the antigenic profiles of Northeastern PCV-2 strains were characterized. The Jameson–Wolf antigenic index revealed dissimilarities in antigenic profiles among PCV-2 clusters. The antigenic indexes of PCV-2 isolates of this study may help to compare or find out recombination events in PCV-2 in India in future as well as it may help to standardization ELISA based protocols on the basis of constant and variable antigenic site.

Present intensive pig production systems are followed; hence, it is possible for pigs to be simultaneously infected with two or more viral pathogens (Blomström *et al.,* 2010). PCV-2 and PPV both are economically important viruses that cause reproductive and/or respiratory failure, in pigs. Furthermore, a definitive aetiological diagnosis based on clinical signs is difficult; in case of multiple infections, because they can have some clinical signs in common (Jiang *et al.,* 2010). So, under such conditions, rapid and reliable detection of these viruses is essential for epidemiological surveillance and disease management. Especially, the rapid detection of infected animals would reduce the potential of transmission of the viruses to healthy animals and avoiding the spread of diseases.

The development of a multiplex PCR method is a complex task mainly due to presence of more than one set of primer pairs in the same reaction mix that may limit the sensitivity or specificity of reaction. In our present study, standardization of duplex PCR strategy in normal conventional PCR as well as in Real-time PCR would help simultaneous detection of PCV-2 and PPV. Detection of PCV-2 in conventional PCR was 10 fold lower as compared to Real time PCR. SYBR Green real-time PCRs coupled to melting curves analysis provides a reliable method for the detection and differentiation of nucleic acid targets (Tam *et al.,* 2009) and in our present study, PCV-2 and PPV was detected with high specificity through well differentiate melt curve (difference in T_m was almost 5^0c) analysis. This multiplex PCR has the potential for considerable saving of time and effort within the laboratory without or less compromising in specificity and sensitivity of the virus detection.

Conckusion

Present field studies represent that PCV-2 and PPV persist in tissues and blood in the presence of high antibody titer values of PWMS suspected animals. Presence of three globally reported genogroups i.e., PCV-2a, 2b, 2d and in addition a variant/emergent strain would help to promote viral genetic diversity. Recombinant strains of PCV-2 reported from Northern India had originated from recombination events (Anoopraj *et al.,* 2015). In addition porous inter and international border with inconvenient animal movement increases the chances of spreading of virus to other parts of country. Prevalence of highly pathogenic PCV-2b genotype would help to generate PWMS in porcine populations of Northeastern region. Amino acid substitution in capsid protein of PCV-2 and PPV may change the virulence and pathogenicity of these viruses. The multiplex PCR assay provides a sensitive tool for simultaneous and rapid detection PCV-2 and PPV infections in pigs and it could be a good alternative for a diagnostic laboratory with limited economic resources. The Main conclusion of the study was the relative high prevalence of both these viruses in North eastern India and genetic variations observed in PCV-2. The importance of the study with regard to concomitant infections and changes in viral sequences necessitate a greater surveillance strategy for PWMS in this region.