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Biological and antigenic properties of the major envelope glycoprotein of porcine reproductive and respiratory syndrome virus

par

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SUMMARY

"Mystery swine disease", later renamed porcine reproductive and respiratory syndrome (PRRS), was recognized as a new disease entity of swine since 1987 in the US (Hill, 1990). Concurrently, a syndrome similar to PRRS had been reported in the province of Québec (Bilodeau *et al.*, 1991a & 1991b, Martineau *et al.*, 1991). The disease was characterized by abortion and infertility of sudden onset, the birth of weak or dead piglets and increased mortality in young pigs associated with respiratory problems. In most herds, response to treatment was poor and the producers learned to allow the disease run its course, which generally persisted for two to four months. Soon after the appearance of a porcine epidemic with identical clinical symptoms in Europe, the causative agent of PRRS was isolated in 1991 and Koch's postulate was fulfilled (Terpstra *et al.*, 1991; Wensvoort *et al.*, 1991b). The newly isolated pathogen designated as Lelystad virus, was found to be genomically and morphologically related to members of the genus *Arterivirus* (Conzelmann *et al.*, 1993; Meulenberg *et al.*, 1993b), notably equine arteritis virus (EAV) and lactate dehydrogenase-elevating virus (LDV). A morphologically and serologically related virus was also isolated from affected swine herds in Canada and the US (Christianson *et al.*, 1992; Collins *et al.*, 1992b; Dea *et al.*, 1992b).

PRRSV infection is highly transmissible and produces a persistent infection. The combination of these two aspects suggests that PRRSV is potentially a ubiquitous endemic disease and indeed, this is one of the most prominent characteristics of PRRS. Since 1987, PRRSV infection spread rapidly through domestic swine populations in North America, Europe and Asia (Bautista *et al.*, 1993a, Chang *et al.*, 1993, Dea *et al.*, 1992d, Wensvoort *et al.*, 1992a). The extent of PRRSV infection prevalence in swine producing areas of Canada is not recognized, but extrapolation of the US data indicating that 82.7% of the farms tested are found positive (Bautista *et al.*, 1993a), may provide a good indication of the economic impact of PRRSV infection in Canada. As is shown in Table 1, after cattle, swine production is the second largest animal industry in Canada.

This is not indicated in Table 1

Also, swine farming is the most important animal production in the province of Québec, with the largest swine population across Canada. Therefore the economic importance of PRRS cannot be over-estimated. As will be explained in greater detail in the next chapter (see under immunization), in spite of remarkable achievements relating to understanding of virology and epidemiology of PRRSV, no appropriate vaccine is yet available for prophylaxis of PRRS and this presents one of the most difficult problems confronting the swine industry.

Ever since PRRSV was characterized and its relation to Arteriviruses was determined, it was sought to establish if data acquired from other Arteriviruses were applicable to PRRSV. The role of ORF5-encoded envelope glycoprotein (G_L) of EAV and it's counterpart VP3 of LDV in virus neutralization and localization of the neutralizing domains of the above mentioned proteins had already been established before beginning research work concerning the present thesis. The above information were persuasive to find out if the ORF5-encoded major envelope glycoprotein of PRRSV had the same role and if so, is it an appropriate candidate for a subunit recombinant type vaccine. Therefore, the objectives of our research work were:

1- To establish the role of the ORF5-encoded major envelope glycoprotein (GP_5) of PRRSV in virus neutralization through characterization of specific monoclonal antibodies.

2- To study the genomic variability of ORF5 and to establish if such variability is reflected in antigenic variability of GP_5.

3- To establish if pigs immunized with the ORF5-encoded recombinant protein develop neutralizing antibodies and are subsequently protected against a virus challenge and to determine the protein specificity of neutralizing antibodies present in pig sera.

4- To develop truncated ORF5 genomic libraries and expression of the corresponding genes in procaryotic expression systems, in order to define the neutralizing domains of the GP_5.

The realization of the first three objectives were subject of four publications already accepted for publication in scientific periodicals and are described in chapters two to four respectively. The objective described under four was partially accomplished and the materials, including the recombinant truncated proteins are available for subsequent research studies. Since this part of our work was not sufficient for an independent publication, the respective methodology and results are described in chapter four of this thesis and its submission in form of an article remains pending on the follow-up work on this part of the project. The overall results of these research works would be crucial to designate strategies for developing a subunit recombinant type vaccine for prophylaxis of PRRSV infection which is essential for defying this costly porcine epidemic.

Briefly, by characterizing MAbs specific to the GP_5 of PRRSV, we have established that as in other Arteriviruses, the ORF5-encoded glycoprotein of PRRSV is associated with neutralizing activity (Part II, Article 1). As with other strains of PRRSV, the GP_5 of the Canadian isolates of PRRSV are genomically variable. Furthermore the IIF reactivity pattern of these strains with the strain-specific anti-ORF5 rabbit sera or the anti-GP_5 MAbs revealed their antigenic variability (Part II, Article 2). Immunization by an *E. coli* expressed recombinant protein encoded by the ORF5, or DNA vaccination by a mammalian plasmidic expression vector encoding the GP_5 of PRRSV, revealed that immunization with the native form of GP_5 elicits neutralizing antibodies in pigs and confers partial protection against a massive viral challenge (Part II, article 3). However, DNA immunization was not sufficient to inhibit viral persistence in the lungs and immunization with the *E. coli*-expressed recombinant protein may have potentiated virus replication in the lungs through an ADE phenomenon. By studying the

reactivity pattern of porcine convalescent sera with different PRRSV recombinant proteins expressed in *E. coli*, we have also established that neutralizing anti-PRRSV antibodies present in porcine convalescent sera are directed to the GP_5 (Part II, Article 4, B. Pirzadeh as the second contributing author). Furthermore, preliminary results obtained from reactivity of several pools of neutralizing PRRSV convalescent sera with the truncated ORF5 encoded proteins by WB indicate that these sera contain antibodies which are directed against continuous epitope(s) located at the carboxy-terminal part of GP_5. This segment of the protein is formed by 42 aa and shows a high amphipathic profile which favors formation of α helix and immunodominant epitope formation.

Our overall results indicate that the GP_5 of PRRSV is a good candidate for a recombinant subunit type vaccine, provided that the hypervariable areas of the protein would not be implicated in neutralizing domains of the protein. The follow-up of our research work involving the reactivity of MAbs or the neutralizing porcine convalescent sera with the truncated proteins described in the Part II, Chapter 5 of the present thesis should provide a convenient answer to this question.

Table 1: Pig and Cattle Population of Canada*

	Pig Population			Cattle Population§		
	Total	Sows	All Others†	Total	Dairy	All Others‡
CANADA	**11,040**	**1,089**	**9,951**	**14,894**	**3,514**	**11,370**
Quebec	3,444	318	3,113	1,440	721	719
Ontario	2,831	298	2,535	2,286	856	1,430
Manitoba	1,777	178	1,599	1,355	226	1,129
Saskatchewan	757	72	685	2,724	333	2,391
Alberta	1,730	174	1,556	5,942	1,055	4,887
British Columbia	174	20	154	814	196	618
Other Provinces	327	31	296	332	126	206

* Data extracted from Agriculture Profile of Canada, Catalogue No. 93-356-XPB, consensus of May 1996, a Statistics of Canada publication.

† Including boars used for breeding.

‡ Including heifers.

§ Data is reported for comparison purposes only.

Résumé

Le syndrome reproducteur et respiratoire porcin (SRRP) est une maladie virale d'importance économique considérable, caractérisée par des signes d'hyperthermie et d'anorexie, accompagnés par des problèmes reproducteurs chez les truies de toutes parités, et des problèmes respiratoires chez les porcs de tous âges. Le SRRP est apparu dans plusieurs états américains de même qu'au Canada au cours de l'année 1987. La maladie fut rapportée en Allemagne vers la fin de l'année 1990 et se propagea rapidement dans l'Europe occidentale. Dès 1994, la présence de l'infection avait été officiellement reconnue dans les élevages de porc de plus de 16 pays de l'Amérique, l'Europe et l'Asie.

La sévérité des signes cliniques est très variable selon le statut immunitaire des animaux et les souches virales en cause: il peut s'agir d'une atteinte asymptomatique, d'une infection chronique se manifestant par une baisse de la productivité (retard de croissance, problème d'infertilité), ou d'une atteinte plus manifeste où les problèmes respiratoires peuvent être responsables de mortalités, de hauts taux de condamnation à l'abattage et d'un nombre très accru d'avortements tardifs ou de pertes majeures de jeunes porcelets.

Chez les porcelets, les lésions induites par le virus sont observées principalement au niveau de l'épithélium respiratoire et se traduisent par une pneumonie de type interstitielle à prolifération. En examen microscopique, ce type de pneumonie se caractérise par une infiltration des septa inter-alvéolaires par des cellules inflammatoires mononucléées comme les lymphocytes et les macrophages. Aucune lésion n'est détectable chez les foetus avortés.

Le virus en cause (VSRRP) possède des similarités morphologiques, physico-chimiques et moléculaires avec les membres du genre *Arterivirus,* incluant le virus de l'artérite équine (VAE), le virus de l'élévation de la déshydrogénase lactique (VEDL) des souris et le virus de la fièvre hémorragique simienne (VFHS). Les

particules virales mesurent 45-55nm de diamètre et contiennent une nucléocapside icosahédrique de 25-35nm entourée par une enveloppe lipidique. Les *Arterivirus* infectent des sous-populations de macrophages, une particularité partagée par le VSRRP qui *in vivo* se réplique exclusivement dans les macrophages alvéolaires du poumon (MAP)s. Jusqu'à présent, seules les lignées cellulaires MARC-145 et CL2621, toutes deux dérivées des cellules épithéliales de rein de singe, s'avèrent permissives au VSRRP.

Le genre *Arterivirus* est classé dans la famille *Arteriviridae*, à l'intérieur de l'ordre *Nidovirales*. Ce nouvel ordre comprend aussi la famille *Coronaviridae*, dans laquelle sont inclus les genres *Coronavirus* et *Torovirus*. Ce classement fut établi sur la base des nouvelles connaissances acquises en regard de la biologie moléculaire des *Arterivirus* notamment le mécanisme de transcription qui est similaire à celui des membres de la famille *Coronaviridae*.

Le VSRRP est un virus à ARN simple brin, de polarité positive et d'environ 13 kb de longueur. Cet ARN génomique contient huit cadres de lecture (ORFs) qui par l'intermédiaire des six ARNm sous-génomiques codent pour les protéines structurales spécifiques du virus. Environ 80% de la longueur du génome en partant de son extrémité 5' code pour l'ORF1, qui est constitué de deux sous-unités ORF1a et ORF1b. L'ORF1a code pour des protéines appartenant à la super famille des protéases et des papaïnes. Les domaines codant pour la polymérase et l'hélicase sont aussi identifiés au niveau de l'ORF1b. Les protéines structurales du VSRRP, la glycoprotéine majeure d'enveloppe (GP_5), la protéine de matrice (M) et la protéine de la nucléocapside (N), sont codées par trois cadres de lecture (ORF5-7) situés à l'extrémité 3' du génome viral. Les données récentes concernant les produits de la traduction des cadres de lecture 2, 3 et 4 de la souche prototype européenne de Lelystad (LV) indiquent que ces protéines sont aussi incorporées dans les particules virales. Les produits de ces gènes sont aussi glycosylés et ont été désignées respectivement GP_2, GP_3 et GP_4. A l'exception des cadres de lecture 4 et 5, les autres cadres de lecture se chevauchent partiellement

(voir le figure 1.3). Étant donné la plus grande abondance de GP_5, M et N comparativement aux protéines GP_2, GP_3 et GP_4, les trois premières sont considérées comme les protéines structurales majeures du VSRRP.

Suite à la caractérisation du VSRRP et à l'identification de son aparenté aux virus du genre *Arterivirus*, nous avons cherché à savoir si les informations acquises sur ces autres virus pouvaient être appliquées au VSRRP. Le rôle au niveau de la neutralisation de la glycoprotéine G_L du VAE, ainsi que de son homologue VP3 du VEDL, toutes deux codées par l'ORF5 du génome de ces virus, avait déjà été établi avant que débutent les travaux faisant l'objet de cette thèse. Ces informations nous ont incité à évaluer le rôle de la GP_5 du VSRRP dans l'induction d'anticorps neutralisants, et en cas d'affirmation, d'établir si cette protéine pourrait être un candidat potentiel pour un vaccin de type sous-unitaire. Ainsi, les objectifs de notre projet de recherche étaient:

1- Par une étude sur la caractérisation d'anticorps monoclonaux (AcMo), établir le rôle de la glycoprotéine majeure du VSRRP, codée par l'ORF5, dans la neutralisation virale.

2- Déterminer si la région ORF5 du génome d'isolats canadiens était sujette à des variations et d'établir si ces dernières avaient des répercussions au niveau antigénique.

3- Établir le rôle de la GP_5 dans l'induction des anticorps neutralisants chez des porcelets immunisés par la protéine exprimée dans un vecteur procaryote ou suite à leur inoculation d'ADN plasmidique porteur du gène. Démontrer le niveau de protection des animaux ainsi immunisés contre une infection expérimentale par le VSRRP. En outre, déterminer la spécificité protéique des anticorps neutralisants, présents dans les sérums de porcs convalescents.

4- Tronquer le gène ORF5 d'une souche de référence du Québec et expression dans un vecteur procaryotique afin de déterminer la topographie des déterminants antigéniques associés à la neutralisation.

La réalisation des trois premiers objectifs a fait l'objet de quatre articles ayant été acceptés pour publication dans des revues scientifiques. Le quatrième objectif a été partiellement accompli, puisqu'une librairie de protéines tronquées recombinantes a été réalisée et pourra éventuellement servir à la poursuite des travaux de recherche. Cette partie de nos travaux ne suffit pas pour une publication indépendante; la méthodologie et les résultats préliminaires sont décrits à la partie 3 chapitre 5 de cette thèse.

Des AcMo dirigés contre la GP_5 ont été obtenus suite aux expériences de fusion entre les splénocytes de souris immunisées par une protéine de fusion GST-ORF5, provenant de la souche québécoise de référence IAF-Klop du VSRRP et exprimée dans *E. coli,* et les cellules d'un myélome murin non-secréteur. La spécificité polypeptidique de ces AcMo produits dans les liquides d'ascite a été confirmée par des expériences d'immunobuvardage et d'immunoprécipitation utilisant d'une part, les préparations virales purifiées par ultracentrifugation isopycnique sur gradients de densité de saccharose, et d'autre part, les protéines de fusion exprimées dans *E. coli* utilisant les vecteurs procaryotiques pGEX-4T et pET-21a. Les profils d'immunofluorescence observés avec les cellules MARC-145 infectées avec la souche virale homologue étaient compatibles avec une localisation de la GP_5 au niveau du réticulum endoplasmique et l'appareil de Golgi. Cinq de ces anticorps inhibaient non seulement l'effet cytopathogène du virus, mais aussi sa réplication au niveau des cellules permissives puisqu'il fut impossible de révéler, par le test d'immunoperoxydase, la présence de la nucléoprotéine du virus dans les cellules infectées en présence de ces anticorps. Compte tenu de la capacité de

ces anticorps de neutraliser le virus *in vitro*, la glycoprotéine majeure (GP_5) du VSRRP apparaît donc comme l'homologue de la G_L de VAE, portant elle aussi les domaines neutralisants. Il été démontré entre autre que les épitopes neutralisants de la GP_5 ne sont pas affectés par l'absence des résidus polysaccharides et qu'il existe aussi des déterminants antigéniques linéaires associés aux domaines neutralisants de la GP_5 (Article 1, Deuxième partie).

Par ailleurs, les ORF5 de neuf isolats du VSRRP associés avec des épisodes aigus ou chroniques de la maladie au Québec et en Ontario ont été séquencés. Les résultats d'analyse des séquences en nucléotides ont révélé que comme les isolats américains du VSRRP, l'ORF5 des souches canadiennes est aussi sujet à des variations. Les produits des ORF5 de trois isolats canadiens, ainsi que la souche vaccinale atténuée d'origine américaine et la souche LV ont été exprimés dans *E. coli* sous la forme de protéines recombinantes fusionnées à la GST, et des sérums hyperimmuns ont été préparés chez des lapins. Les profils de réactivité de ces isolats envers les AcMo dirigés contre la GP_5 de la souche IAF-Klop et les sérums de lapin mono-spécifiques anti-GP_5 des cinq différentes souches étudiées, ont démontré que les variations génomiques observées se traduisent aussi par des changements au niveau de la spécificité antigénique de la GP_5 (Article 2, deuxième partie).

Les résultats des études de protection conférée, suite à l'immunisation génétique des porcelets à l'aide d'un plasmide permettant l'expression de la GP_5 dans les cellules de mammifères, et l'immunisation à l'aide de protéine de fusion GST-ORF5 exprimée dans *E. coli,* ont révélé l'importance de la conformation et probablement de la glycosylation de la GP_5 pour l'établissement d'une réponse immunitaire protectrice. En effet, il a été possible de protéger les porcelets contre l'établissement des lésions pulmonaires sévères et les signes cliniques du SRRP suite à l'immunisation génétique, ce qui ne fut pas le cas des animaux immunisés à l'aide de la protéine exprimée par un vecteur procaryote (Article 3 deuxième partie). Par contre, l'immunisation génétique n'a pas suffi pour éliminer l'infection

virale persistante des voies respiratoires des animaux inoculés avec une dose infectieuse massive du VSRRP. Les résultats obtenus ont démontré que l'immunisation avec la GP_5 recombinante exprimée dans *E. coli* pourrait avoir plutôt contribué à amplifier l'infection au niveau des poumons. Ce phénomène serait dû en partie à la synthèse dans ce cas d'anticorps facilitateurs non-neutralisants qui favoriseraient l'entrée des complexes immuns dans les macrophages alvéolaires par l'intermédiaire des récepteur Fc à leur surface. Les résultats de nos expériences en regard de la réactivité des sérums neutralisants convalescents des porcs envers les différentes protéines recombinantes virales (GST-ORF3, GST-ORF4, GST-ORF5 et GST-ORF7) exprimées chez *E. coli*, ont aussi établi que les anticorps neutralisants présents dans ces sérums sont surtout dirigés contre le GP_5 du VSRRP. En effet une corrélation entre les titres obtenus en ELISA en anticorps anti-GP_5 et les titres en anticorps neutralisants a pu être établie (Article 4, deuxième partie; B. Pirzadeh, deuxième auteur).

En conclusion, ces propriétés de la GP_5 nous indiquent que cette protéine apparaît comme un bon candidat pour l'établissement d'un vaccin de type sous-unitaire en autant que les régions variables n'impliquent pas les épitopes majeurs associés à la neutralisation. Les résultats préliminaires sur la réactivité des protéines tronquées avec les sérums convalescents de porcs neutralisants ont montré que ces sérums contiennent des anticorps dirigés contre la partie carboxyterminale de la protéine (chapitre 4, partie II). Il existe au niveau de cette région de la GP_5 une séquence d'environ 42 aa montrant un profil hautement amphipathique. Ceci pourrait favoriser la conformation hélicoïdale de type α et la formation d'épitopes immunodominants linéaires. Il faut tenir compte que les anticorps spécifiques à la GP_5 sont détectés sept à onze jours après l'infection par le VSRRP, tandis que les anticorps neutralisants ne sont décelés que trois à quatre semaines après l'infection. Bien que ce phénomène pourrait être associé aux anticorps de haute affinité sécrétés plus tardivement au cours de la réponse immunitaire, il ne faut pas rejeter la possibilité que les anticorps précoces soient dirigés contre les domaines immunodominants non-neutralisants de la GP_5. Il est

donc essentiel d'étudier la réactivité des AcMo neutralisants envers la GP_5. La présence au niveau de la GP_5 de domaines impliqués dans le phénomène d'amplification d'infection par les anticorps facilitateurs doit être clarifiée. Il a été suggéré que les déterminants antigéniques associés à la neutralisation virale ne sont pas liés aux domaines impliquées dans le rehaussement de la réplication. Compte tenu de la nature spéculative de ces arguments, il est essentiel d'étudier cet aspect important de l'immunogénicité de la GP_5, afin d'éliminer ces régions de la protéine dans un éventuel vaccin recombinant. Présentement, nos connaissances en regard de la réponse immunitaire à médiation cellulaire contre le VSRRP, et surtout le rôle des protéines structurales du virus dans le déclenchement de telle réponse demeurent très limitées. Cet aspect de la recherche sur la réponse immunitaire contre l'infection par le VSRRP mérite d'être étudié en profondeur.

Malgré l'importance des connaissances acquises sur la biologie moléculaire et l'épidémiologie du VSRRP, quelques aspects élémentaires de cette infection virale demeurent obscurs. À titre d'exemple, la pathogénèse des problèmes reproducteurs associés à cette infection ne peut être expliquée si on ne considère que les cellules permissives à la réplication du virus et les organes affectés par les lésions virales. L'hyperthermie par elle-même n'explique pas la pathologie de ces problèmes reproducteurs, incluant les avortements, les mortalités embryonnaires et les problèmes d'infertilité. L'hypothèse selon laquelle les problèmes reproducteurs puissent être la conséquence de problèmes endocriniens associés à des lésions du système nerveux central est plausible, mais des études approfondies sont nécessaires pour la vérifier. Comme dans le cas d'autres virus responsables de syndrome d'immunodéficience chez les mammifères, le VSRRP infecte de façon sélective des populations cellulaires du système immunitaire. En outre, le SRRP conduit aussi à une infection de type persistante où on retrouve un phénomène d'apoptose et une baisse du rapport des cellules $CD4^+/CD8^+$ durant la phase aiguë, et qui est transmissible par le sperme infecté. L'effet à long terme de cette infection virale sur le système immunitaire des porcs exige une étude approfondie.

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List of scientific publications abbreviated in References

AASP Newsletter.:	American Association of Swine Practitioners Newsletter
Am. J. Vet. Res.:	American Journal of Veterinary Research.
Ann. Rech. Vet.:	Annales de la Recherche Vétérinaire.
Anal Biochem.:	Analytical Biochemistry.
Arch. Virol.:	Archives of Virology.
Can. J. Vet. Res.:	Canadian Journal of Veterinary Research.
Can. Vet. J.:	Canadian Veterinary Journal.
J. Am. Vet. Med. Assoc.:	Journal of American Veterinary Medical Association.
J. Biol. Chem.:	Journal of Biological Chemistry.
J. Chin. Soc. Vet. Sci.:	Journal of Chinese Society of Veterinary Science.
J. Clin. Microbiol.:	Journal of Clinical Microbiology.
J. Exp. Med.:	Journal of Experimental Medicine
J. Gen. Virol.:	Journal of General Virology.
J. Immunol.:	Journal of Immunology.
J. Infec. Dis.:	Journal of Infectious Diseases.
J. Mol. Biol.:	Journal of Molecular Biology.
J. Vet. Diagn. Invest.:	Journal of Veterinary Diagnostic and Investigation.
J. Vet. Med. B.:	Journal of Veterinary Medicine of Belgium.
J. Vet. Med. Sci.:	Journal of Veterinary Medicine Science.
J. Virol.:	Journal of Virology.
J. Virol. Methods:	Journal of Virological Methods.
Proc. AASP.:	Proceedings of the American Association of Swine Practitioners.
Proc.CCLWAD.:	Proceedings of the Conference of Canadian Laboratory Workers in Animal Diseases.

List of abbreviations used in the text

aa: Amino Acid.

AcMo: Anticorps Monoclonal

ADE: Antibody Dependent Enhancement.

ATCC: American Type Culture Collection.

BCV: Bovine Coronavirus.

BEV: Berne Virus.

BFS: Bovine Fetal Serum.

BRV: Breda Virus.

BUDR: 5-bromo-2-deoxyuridine.

CCV: Canine Coronavirus.

CD: Cluster of Differentiation.

cDNA: Complementary Deoxyribo Nucleic Acid.

CNS: Central Nervous System.

COS: CV1 defective Origin of SV40

CPE: Cytopathogenic Effect.

DMEM: Dulbecco's Modified Essential Medium.

DNA: Deoxyribo Nucleic Acid.

DTT: Di-Thio-Treitol.

EAV: Equine Arteritis Virus.

EDTA: Ethanol Diamine Tetra-Acetic acid.

ELISA: Enzyme Linked Immuno-Sorbent Assay.

EMCV: Encephalomyocarditis Virus.

EndoH: Endo-β-N-acetylglucosamine.

FIPV: Feline Infectious Peritonitis Virus.

FITC: Fluorescein Iso-Thio-Cyanate.

GST: Glutathione Sulfo-Transferase.

HAAT: Hyperthermie-Anorexie-Avortement de la Truie.

HCV: Human Coronavirus.

HEV: Hemagglutinating Encephalomyelitis Virus.

HIV: Human Immuno-deficiency Virus

IBV: Infectious Bronchitis Virus.

IFA: Immunofluorescent Assay

Ig: Immunoglobulin.

IIF: Indirect Immuno-fluorescent.

IPMA: Immunoperoxidase on Monolayer Assay.

IPTG: Iso-Propyl-β-D-Thio-Galactopyranoside.

kb: Kilo-base.

kbp: Kilo-base pair.

kDa: kilo Dalton.

LDV: Lactate Dehydrogenase-Elevating Virus.

LV: Lelystad Virus.

M-CSF: Macrophage-Colony Stimulating Factor

MAb: Monoclonal Antibody.

MARC: Meat Animal Research Center.

MHV: Murine Hepatitis Virus.

MLV: Modified Live Vaccine.

MOI: Multiplicity of Infection

M_r: Relative Molecular Mass.

NK: Natural Killer.

nt: Nucleotide.

ORF: Open Reading Frame.

PAM: Porcine Alveolar Macrophage.

PBMC: Peripheral Blood Mononuclear Cell.

PBS: Phosphate Buffered Saline.

PCR: Polymerase Chain Reaction.

PEARS: Porcine Epidemic Abortion and Respiratory Syndrome.

PEDV: Porcine Epidemic Diarrhea Virus.

PID: Post-Infection Day.

PMSF: Phenyl Methyl Sulfonyl Fluoride

PNGaseF: Endoglycosidase F.

PPV: Porcine Parvovirus.

PRCV: Porcine Respiratory CoronaVirus.

PRRS: Porcine Reproductive and Respiratory Syndrome.

RCV: Rat Coronavirus.

RIPA: Radio-Immuno-precipitation Assay.

RT: Reverse Transcription.

SDRP: Syndrome Dysgénésique et Respiratoire du Porc.

SDS-PAGE: Sodium Dodecyl Sulfate-Poly-Acrylamide Gel Electrophoresis.

SHFV: Simian Hemorrhagic Fever Virus.

SIRS: Swine Infertility and Respiratory Syndrome.

SN: Serum Neutralization.

SPF: Specific Pathogen Free.

SRRP: Syndrome reproducteur et respiratoire porcin

TCID: Tissue Culture Infectious Dose.

TCV: Turkey Coronavirus.

TGEV: Transmissible Gastro Enteritis Virus.

VN: Virus Neutralization.

VSRRP: Virus du syndrome reproducteur et respiratoire porcin

Proc.CRWAD:	Proceedings of Conference for Research Workers in Animal Diseases
Proc. Int. Pig. Vet. Soc.:	Proceedings of International Pig Veterinary Society.
Proc. Int. Vet. Immunol.:	Proceedings of Inter-Veterinary Immunology.
Proc.Natl.Acad.Sci.USA.:	Proceedings of the National Academy of Sciences of the USA.
R.D.K.J. Agri. Sci.:	Republic Democratic of Korea Journal of Agricultural Science.
Res. Vet. Sci.:	Research in Veterinary Science.
Vet. Microbiol.:	Veterinary Microbiology.
Vet. Pathol.:	Veterinary Pathology.
Vet. Q.:	Veterinary Quarterly
Vet. Rec.:	Veterinary Records.
Vet Res.:	Veterinary Research.
Viral Immunol.:	Viral Immunology.
Virus Res.:	Virus Research.

To my dear Mahine.

PART I

REVIEW OF LITERATURE

1. Porcine reproductive and respiratory disease syndrome

1.1 History

During 1987, a new swine disease of unknown etiology emerged in North America and rapidly spread through the pig population. The disease outbreak was first reported in North Carolina, Minnesota and Iowa (Collins *et al.*, 1991b; Hill 1990; Keffaber 1989), but soon it became evident that the same clinical condition also occurred in Canada, particularly in the province of Québec (Bilodeau *et al.*, 1991a and b; Dea *et al.*, 1990 and 1992a). The disease was characterized by occurrence of both reproductive and respiratory signs but since no known swine pathogen could be implicated in most cases, the syndrome was referred to as "mystery swine disease" (Keffaber, 1989). Initially referred to as "Le syndrome d'hyperthermie-anorexie-avortement de la truie" (HAAT) in the province of Québec (Martineau *et al.*, 1991), other terms such as "Porcine Epidemic Abortion and Respiratory Syndrome (PEARS)" (Wensvoort *et al.*, 1991a), "Swine Infertility and Respiratory Syndrome (SIRS)" (Collins *et al.*, 1991a), "Blue Ear Disease of Pig" (White, 1991; Paton *et al.*, 1991), "le syndrome dysgénésique et respiratoire du porc (SDRP)" (Albina *et al*, 1992b), "abortus blauw" (Terpstra *et al.*, 1991) which were mostly descriptive of clinical symptoms of the disease were used by various authors to refer to the newly emerged clinical entity. The term "Porcine Reproductive and Respiratory Syndrome" (PRRS) was adapted for the disease in 1992 at the First International Symposium on SIRS/PRRS in Minnesota, USA.

In 1990, a syndrome similar to PRRS was reported in Münster, Germany (Lindhaus & Lindhaus 1991; Ohlinger *et al.*, 1991) which was followed by similar clinical cases reported from other European countries including France (Baron *et*

al., 1992), Spain (Plana-Durán *et al.*, 1992a and b), the Netherlands (Cromwijk 1991; De Jong *et al.*, 1991) and the United Kingdom (Paton *et al.*, 1991). PRRS has been officially recognized in 16 countries from three different continents *i.e.* North America, Europe and Asia (Chang *et al.*, 1993; Meredith, 1995; Shimizu *et al.*, 1994; Shin *et al.*, 1993).

1.2 Isolation of Lelystad Virus and fulfillment of Koch's postulate

Soon after the appearance of the "mystery swine disease" in The Netherlands, a new virus was isolated from clinically affected pigs and the SPF pigs experimentally exposed to sick animals (Wenswoort *et al.*, 1991b). The virus was found to be unrelated to other known porcine pathogens and designated Lelystad virus (LV). Pregnant sows experimentally exposed to the newly identified virus propagated on porcine alveolar macrophages (PAM), developed clinical signs similar to the mystery swine disease including blue discoloration of the ears, fever, respiratory distress and abortion. The Lelystad virus was isolated from aborted fetuses or affected piglets that died shortly after birth (Terpstra *et al.*, 1991). Isolation of a virus relevant to PRRS (isolate ATCC VR-2332) and experimental reproduction of the disease in gnotobiotic pigs with the same isolate was reported in the US (Christianson *et al.*, 1992; Collins *et al.*, 1992b). An enveloped virus, morphologically similar and serologically related to LV and ATCC VR-2332, was also isolated the same year from affected pigs in Québec (Dea *et al.*, 1992b and c).

1.3 Clinical disease

Initially, PRRS was characterized as an acute outbreak of reproductive failure in sows of any parities (Keffaber 1989). At present the clinical symptoms of PRRS have remained unchanged. Early signs in breeding sows consist of transient anorexia, listlessness and transient fever (39.7-41.8°C) that may be detected in more than 20% of the animals and may persist during one week. The reproductive

failure is characterized by late-term abortions, early farrowing (107-112 days of gestation), increased stillbirths, mummified fetuses, smaller than normal and weak born pigs (Dea *et al.*, 1992c and d). Stillborn fetuses in affected litters are often autolysed, edematous and have tan-brown discoloration of the skin. Minor pulmonary lesions are sometimes observed in lungs of these piglets (Lager *et al.*, 1994). Often the weak piglets do not nurse properly which may lead to agalactia in sows and ultimately to more starved and crushed piglets (Ahl *et al.*, 1992; Hopper *et al.*, 1992). Affected sows may also develop a blue discoloration of the ears, teats, snout, ventral cervical skin, vulva and abdomen. The recovery in affected sows is generally accompanied by delayed return to estrus and poor conception rates (up to 50 % reduction). Post recovery breeding performance may rarely become normal and remain consistently below pre-epidemic level in majority of cases (Keffaber, 1989).

Respiratory syndromes are mainly observed amongst grower-finisher pigs. The disease may be confused with an influenza-like illness and clinical signs include anorexia, pyrexia (40°-41° C), labored abdominal respiration, sneezing, nasal discharges, eyelid oedema, conjonctivitis, thumping and mouth breathing. Central nervous signs such as lateral recumbency, paddling and vomiting may occasionally be seen. Chronic respiratory form of the disease generally results in reduced weight gain and increased mortality (Goyal, 1993; Magar *et al.*, 1994; Wensvoort, 1993).

In contrast to reproductive disease, clinically overt respiratory disease is difficult to be experimentally reproduced (Van Reeth, 1997; Zimmerman *et al.*, 1997b). Under various experimental conditions, different field isolates of PRRSV have been administered oronasally to 1-14 week old gnotobiotic, specific pathogen free (SPF) or conventional pigs. Most experimental infections failed to produce overt disease and a transient fever was the most consistent and prominent clinical change (Albina *et al,* 1994; Paton *et al,* 1992; Plana Duran *et al* 1992a; Pol *et al,*

1991; Ramos *et al,* 1992). More or less severe respiratory disease has been observed with some US isolates (Halbur *et al,* 1993 & 1995; Rossow *et al,* 1995). Inoculation with some other US isolates however remained without clinical signs (Halbur *et al.,* 1995; Mengeling *et al.,* 1996). Most research groups are of opinion that uncomplicated PRRSV infection is mostly unapparent and does not affect productivity. In the field as well, PRRSV infection may be subclinical and so far a significant effect on weight gain during the acute phase of the disease is not demonstrated. Reports from Europe indicate that seroconversion of feeder pigs was not associated with disease (Houben *et al* 1995). Also in the US, the seroconversion to PRRSV has been reported on farms without any obvious clinical signs, leading some researchers to suggest that PRRS is probably over diagnosed by practioners (Cho *et al* 1993). The latter phenomenon is believed to be related to the virulence of PRRSV strains (Halbur *et al.,* 1996; Meng *et al.,* 1996) and the immune condition of the animals used in experimental infection assays.

1.4 Pathological findings

1.4.1 Macroscopic lesions

Apart from necrotic foci detectable on placenta (Pejsak *et al.,* 1997), no macroscopic lesion can generally be detected in aborting sows. Fetuses, still-borns and partially mummified fetuses are typically seen in early stages of PRRS outbreaks. In piglets, an enlargement of the heart and some changes in the blood vessels suggestive of local congestion may be visible. An increased amount of exudate may be found in the thorax. Gross lesions are confined to the respiratory tract and thoracic cavity. Portions of the lungs are tan and partly collapsed, with occasional anteroventral areas of congestion and consolidation. The mediastinal lymph nodes are enlarged and congested. Adherence of the pleura to the thoracic cage is sometimes observed, with slight accumulation of non-suppurative exudate within the thoracic cavity (hydrothorax) and pericardium (hydropericardium). No

other remarkable gross lesions are observed in the other organs. Pulmonary hepatisation and glandular aspect at lung section accompanied by mild tumefaction of mediastinal lymph nodes may also be observed (Bilodeau *et al.*, 1991b; Dea *et al.*, 1992d; Rossow *et al.*, 1994a and b).

1.4.2 Microscopic lesions

Degeneration of nasal mucosa epithelial cells characterized by loss of ciliated epithelium and desquamation of superficial epithelial layer has been described in young pigs (Pol *et al.*, 1991). Lymphocyte depletion is observed in the spleen, thymic cortex, tonsilar crypts and mesenteric lymph nodes. The spleen may also contain degenerated ellipsoids characterized by karyorrhexis and vacuolization. The affected pigs develop intense interstitial pneumonitis, characterized by hyperplasia of bronchiolar epithelium and pneumocytes type II of the alveolar endothelium, perivascular cuffing, lymphomononuclear cells infiltration and thickening of alveolar septa. Other lesions are generally confined to the lungs and consist of macrophage infiltration, pyknotic cell debris and protein rich exudate in the lumen of large bronchi and bronchioli, a peribronchiolar and perivascular lymphomononuclear cells infiltration, the presence of lymphomononuclear cells within the alveolar lumen with hyperplasia of type II pneumocytes, mononuclear cells invasion and presence of pyknotic cells in alveolar septa. Subacute mononuclear encephalitis and perivasculitis, consisting of macrophages, lymphocytes, plasmocytes surrounding small vessels has also been reported (Collins *et al.*, 1992a; Dea *et al.*, 1992d).

While virus isolation from central nervous system (CNS) of PRRSV-infected pigs has been unsuccessful, presence of PRRSV antigens has been demonstrated in CNS by immunohistochemical methods and microscopic lesions have been described (Rossow *et al.*, 1996, Thanawongnuwech *et al.*, 1997). Histological examination of the tissues from the weanling gnotobiotic pigs experimentally

infected with PRRSV consisted of marked lymphohystiocytic encephalitis, gliosis and severe necrotizing vasculitis. Association of PRRSV antigen with neurovascular lesions can also be demonstrated by cytoplasmic staining of microglia-like cells, mononuclear cells within the walls of cerebral arteries, perivascular and meningeal mononuclear cells in the brain section.

1.5 Epidemiology

1.5.1 Prevalence

Today PRRSV infection is ubiquitous in swine producing areas of the world, including Canada and the US, Central America, Europe and Asia (Baron *et al.*, 1992; Chang *et al.*, 1993; Correra Girón *et al.*, 1994; Shin *et al.*, 1993). In the US, a retrospective serological study showed that PRRSV infected Iowa swine population between 1980 and 1985. Growing proportion of positive sera indicate rapid propagation of the disease from 0% in 1980 to 9.6% in 1985 and 51.7% in 1989 (Bautista *et al.*, 1993a). Accurate reports concerning prevalence of the infection are only available from the US, where 40-60% of herds are estimated to be infected. The percentage of infected farms is highly variable, ranging from 0 to 80% in different States (Albina 1997). In Europe, PRRSV infection is believed to have affected more than 50% of the farms. The prevalence is highly regional depending on pig density of a given area (Le Potier *et al.*, 1997).

1.5.2 Mode of transmission

PRRSV is perhaps the most infectious agent of all swine pathogens. Pigs exposed to 10 $TCID_{50}$ by intranasal or intramuscular routes become infected (Zimmerman *et al.*, 1997b). The primary transmission route is via close contact between carrier and susceptible pigs, through nose-to-nose or by contact with urine and feces.

While exposure to PRRSV via airborn aerosol has been proposed as the primary route of transmission, no direct evidence exists for aerosol transmission of PRRSV. Farms located in the vicinity (less than 0.5 km) of a PRRS outbreak are 20 times more at risk than those situated outside the 1 Km radius (Le Potier *et al.*, 1997). Furthermore, transmission via insemination by semen collected during acute phase of the disease is now well documented (Swenson *et al.*, 1995).

1.5.3 Viral natural reservoirs

As other arteriviruses (Plagemann & Moenning 1992), PRRSV can produce a persistent infection (Bilodeau *et al.*, 1994; Wills *et al.*, 1997a). Several weeks after apparent recovery from acute phase of infection, pigs continue to shed virus via several routes. While PRRSV cannot be isolated from fecal samples of infected pigs, viral genome is detectable by reverse transcription-polymerase chain reaction (RT-PCR) in fecal specimens up to 21 post-infection days (PID). Isolation of PRRSV from oropharyngeal samples for up to 157 days after experimental infection suggests that the virus may persist in the upper respiratory tract, perhaps the palatine tonsils (Wills *et al.*, 1997a). Clinically healthy carriers may therefore be considered as an important source of virus.

Infected boars shed virus in semen as long as 43 PID (Christopher-Hennnings *et al.*, 1995a and b; Swenson *et al.*, 1994). Using RT-PCR, viral RNA can be detected in semen of experimentally-infected boars 92 PID (Christopher-Hennnings, *et al.*, 1995a). Transmission of PRRSV to female using artificial insemination with PRRSV-infected semen has been demonstrated. At present, the source of PRRSV in semen is not known. Seminal shedding can occur in absence of detectable viremia, suggesting that the phenomenon is not due to "spill-over" of free virus from the blood. Virus isolation from bulbourethral gland of an experimentally-infected boar may indicate virus replication in the reproductive tract

or the accessory lymphatic nodes (Christopher-Hennnings *et al.*, 1995b; Yaeger *et al.*, 1993).

The half life of PRRSV (LV strain) was found to be 140h at 4°C and 3h at 37°C. Furthermore, the virus can be recovered at relatively high titres from various tissues of viremic animals kept for relatively prolonged periods of time at 4°C. The tissues found to contain live virus after storage were muscle, spleen, lymph nodes and serum (Bloemraad *et al.*, 1994; Van Alstine *et al.*, 1993a). Persistence of PRRSV in the environment has been studied in or on 16 fomites including plastic, stainless steel, rubber, alfalfa, wood shaving, straw, corn, swine starter feed, denim cloth, phosphate buffer saline solution, saline G, well water, city water, swine saliva, swine urine and fecal slurry (Pirtle *et al.*, 1996). At ambient temperature (25-27° C), virus did not persist on fomites beyond day zero except for PBS, through day three, saline G through day six, well water through day eight, and city water through day 11. In general, PRRSV appears to be a relatively labile virus which requires a moist environment to avoid inactivation.

Susceptibility of Mallard ducks, guinea fowl and Cornish cross chickens to PRRSV has been reported (Zimmerman *et al.*, 1997a). Recovery of virus five days and more after exposure provided evidence for viral replication in one or more of the systems emptying into the cloaca. Based on the duration of fecal shedding of the virus it is suggested that avian species have variable susceptibility to PRRSV. Transmission of virus among birds as well as transmissibility of avian-passaged PRRSV in swine has been demonstrated, suggesting the involvement of birds in epidemiology of PRRSV. In experimental studies, PRRSV infection of birds was sub-clinical, but their feces could remain infectious for at least 24 days. Rodents are often closely associated with swine farms but cannot be experimentally infected (Hooper *et al.*, 1994). Natural infection with PRRSV of wild boars was indirectly indicated by a sero-epidemiological survey carried out in Germany (Oslage *et al.*, 1994) and confirmed in France (Albina, 1997). However, there is

no report of clinical signs of PRRS infection in wild boars and no substantiation of their potential role in transmission of PRRSV to domestic pigs.

2 Characteristics of PRRSV

2.1 Classification

Based on a joint proposal by the Coronavirus and Arterivirus Study Groups during the Xth International Congress of Virology held in Jerusalem in August 1996, the International Committee for Taxonomy of Viruses (ICTV) placed PRRSV in the genus *Arterivirus,* family *Arteriviridae*, within the recently created order *Nidovirales* (Cavanagh, 1997). This newly created order (Figure 1.1) comprises *Coronaviridae* family which includes genera *Coronavirus* and *Torovirus* (de Vries *et al.,* 1997; Pringle *et al.,* 1996). Members of the arteriviruses were previously classified within the Togaviridae family (Westaway *et al.,* 1985), but the need for reclassification became obvious after cloning and molecular analysis of the total EAV genome (Den Boon *et al.,* 1991) and part of LDV genome (Godeney *et al.,* 1990). Contrary to togaviruses, arterivirus gene expression does not occur by translation and subsequent processing of polyproteins, but by transcription of multiple subgenomic mRNAs, each encoding one protein. Similar to coronaviruses, arteriviral mRNAs form a 3' coterminal nested set and possess common 5' terminal leader sequence. The large nested-set arrangement of the subgenomic mRNAs was the inspiration for the name of the order, from the Latin *nidus*, nest. Other members of *Arterivirus* genera comprise murine lactate dehydrogenase elevating virus (LDV), equine arteritis virus (EAV) and simian hemorrhagic fever virus. Furthermore the definition order was introduced into virus classification to include families of viruses with similar genomic organisation and replication strategies.

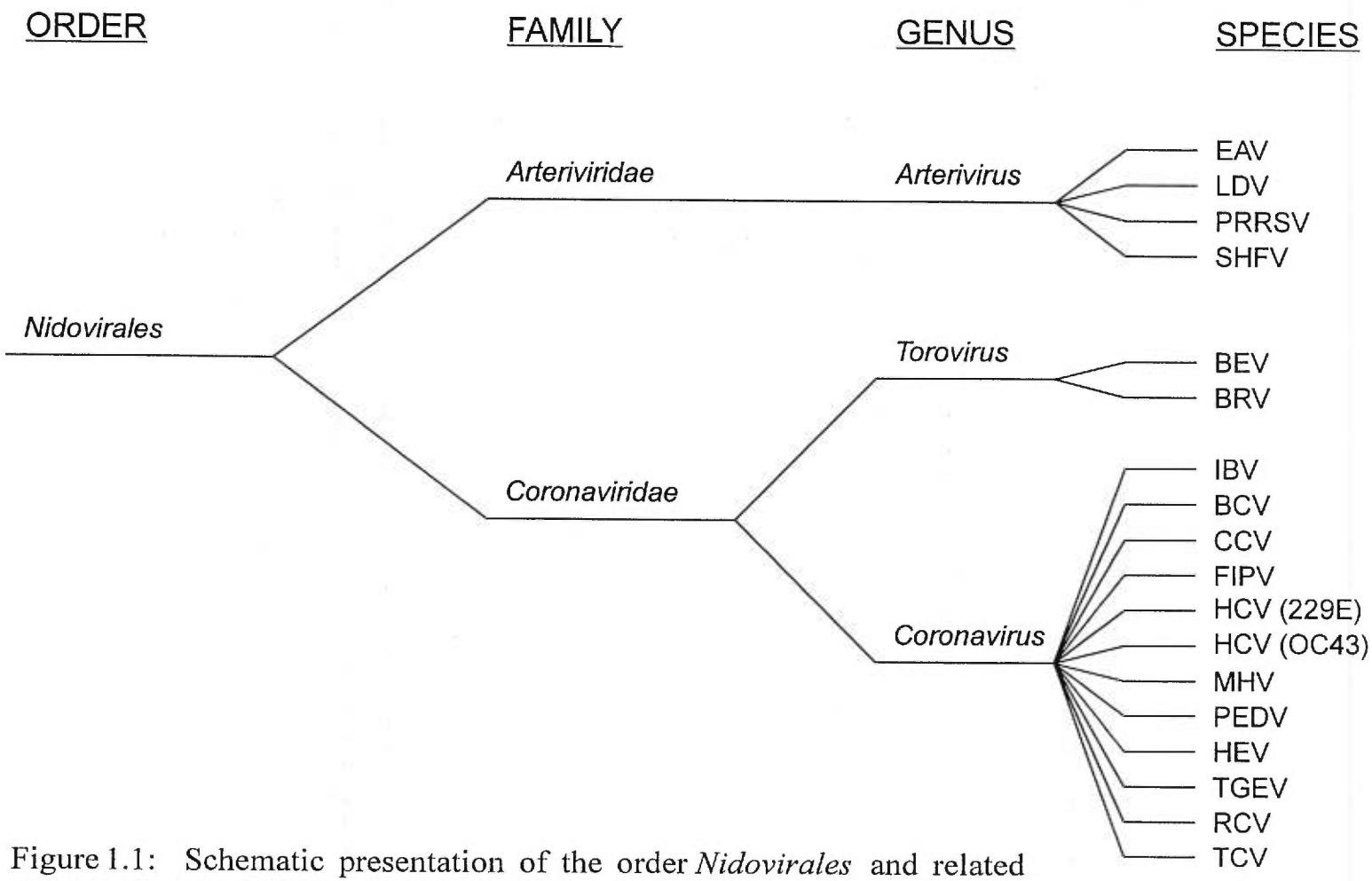

Figure 1.1: Schematic presentation of the order *Nidovirales* and related species (Adapted from Cavanagh, D. 1997 and Murphy & al., editors in Virus Taxonomy 1995)

2.2 Morphological characteristics

The morphological characteristics of PRRSV closely resemble those of arteriviruses namely EAV, LDV and SHFV. PRRSV is a pleomorphic, mostly spherical enveloped virus of 50-70 nm in diameter as estimated by ultra filtration and electron microscopy (Benfield *et al.,* 1992a and b; Mardassi *et al.,* 1994a; Wensvoort *et al.,* 1991b and 1992a), containing an isometric core of 25-30 nm. When PRRSV is propagated in PAMs and purified on cesium chloride (CsCl) gradients, three opalescent bands are obtained. Virus obtained from tissue culture supernatant of CL2621 cell line (appropriation of Boehringer Ingelheim Animal Health Inc., St. Joseph MO) produced a single band. In both cases, the peak infectivity of the purified virus occurs at 1.18-1.19 g/mL. The middle band obtained from PAM-propagated PRRSV corresponding to buoyant density of 1.28-1.31 g/mL contains mostly core particles.

2.3 Isolation in cell cultures and cytopathogenic effect (CPE)

PRRSV was originally isolated on primary cultures of porcine alveolar macrophages (Wensvoort *et al.,* 1991b) and so far, these cells remain the only porcine cells that can effectively be used for viral propagation *ex vivo*. Two other permissive cell lines are MARC-145 cells, (Kim *et al.,* 1993) and CL2621 cells (Bautista *et al.,* 1993b), both derived from monkey kidney MA-104. The CPE on both cell lines consists of appearance of small rounded clumps of cells raising above the remainder of the infected monolayer. Infected cells become pycnotic and detach from the monolayer two to four post-infection days (PID). Infectivity titers of 10^5 to 10^7 $TCID_{50}$/mL are usually obtained from clarified tissue culture supernatants of these cells lines after five to seven serial passages. On PAMs, the CPE is characterized by rounding off, lysis and clumping of the cells. Infected cells showed a bristling cytoplasmic membrane, granulation and usually destruction

of monolayers after 36-48h post-infection (Benfield *et al.*, 1992a; Mardassi *et al.*, 1994a).

2.4 Biochemical characteristics

The compounds 5-bromo-2-deoxyuridine (BUDR) and mitomycin C, as inhibitors of DNA replication, have no effect on PRRSV replication in CL2621 cells. Acidic pH is reported to enhance viral infectivity *in vitro* (Kreutz *et al.*, 1996). Pretreatment of PRRSV with chloroform eliminated infectivity, whereas fluorocarbon treatment had no significant effect (Benfield *et al.*, 1992a). Controversy exists regarding the hemagglutinating properties of PRRSV since it was previously reported as a non-hemagglutinating virus (Benfield *et al.*, 1992a Mardassi *et al.*, 1994a). More recent reports (Jusa *et al.*, 1996 & 1997), indicate that PRRSV can effectively agglutinate mouse erythrocytes and that heparin inhibited hemagglutination. However, virus purification and hemagglutination test procedures used in the above mentioned reports were not identical.

2.5 Genomic characteristics and replication strategy

PRRSV contains a linear, polyadenylated, single stranded, positive-sense RNA genome of approximately 15 kb in size. The two third 5' part of the genome encodes two large overlapping open reading frames (ORF)s, ORF1a and ORF1b which code for polymerase poly-proteins (Meulenberg *et al.*, 1993a).

Most of information on arterivirus polyprotein processing comes from works on EAV (Den Boon *et al.*, 1995, Snidjer *et al.*, 1992; 1994a & b), while only limited data is available for PRRSV and LDV. The ORF1a encodes for a protein with apparent RNA dependent RNA polymerase activity which possesses two papain-like proteinase domains (Meulenberg *et al.*, 1993b). The PRRSV and LDV leader proteinases share 48% sequence identity. ORF1b processing would yield a

protein containing both helicase domain and a zinc finger motif. The genomic RNA functions as the mRNA for the translation of partially overlapping ORFs 1a and ORF1b, the latter being translated through ribosomal frame shifting. The ORF1b encodes the putative polymerase and helicase domains, the latter being situated downstream of the polymerase domain. Overall there is 40% and 25% identity in amino acid sequence of the ORF1a of LV with the corresponding proteins of LDV and EAV. As for the ORF1b, the percentage of amino acid identity is 67% and 38% for EAV and LDV, respectively. Therefore LV appears to be closer to LDV than EAV with respect to ORF1 encoded aa sequence (Figure 1.2).

Analogous to all members of the order *Nidovirales*, multiple subgenomic RNAs are synthesized during virus morphogenesis in infected cells. They all form a 3' coterminal nested set and all contain a leader sequence derived from the 5' end of the viral genome (Figure 1.3). The junction sites, where the leader is fused to the body of the subgenomic RNA, were found to contain a conserved motif of 6 nucleotides UCAACC in LV, but this consensus sequence was absent from subgenomic mRNAs encoding ORFs 3,4 and 5 of the Québec isolate IAF-exp91 (Conzelmann *et al.*, 1993; Meulenberg *et al.*, 1993a; Mardassi *et al.*, 1995). Only the 5' unique regions of the mRNAs which are absent from the next smaller mRNA are translated.

PRRSV genome contains a 3' untranslated sequence downstream of the ORF7. This region consists of 151 nucleotides and a poly A tail of 19-20 bases (Murtaugh *et al.*, 1995, Mardassi *et al.*, 1995). The 3' noncoding region of LV has 114 nucleotides (Meulenberg *et al.*, 1993a). The nucleotides at positions 50-151 of the non-coding region of ATCC VR-2332 and IAF-exp91 strains share a strong homology with those at positions 13-114 of the similar region of LV. The function of this sequence is unknown.

Sequence analysis of two European (LV and PRRSV 10) and two North American (IAF-exp91 and ATCC VR-2385) PRRSV strains revealed that ORFs 2-7

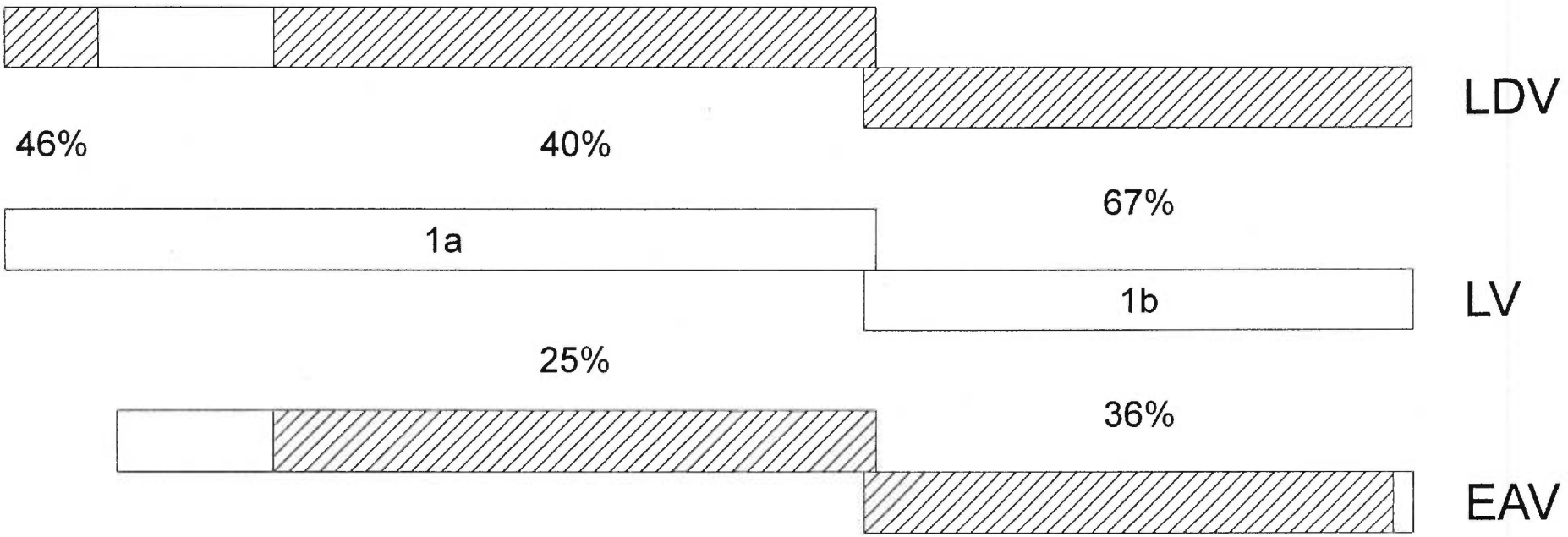

Figure 1.2: Comparison of amino acid sequences of ORFs 1a and 1b of LV, EAV (den Boon et al., 1991) and LDV (Godeny et al., 1993). The percentage of amino acids identity is indicated. The regions of the ORFs of EAV and LDV, which have identity with LV are shaded (Meulenberg et al., 1997b).

are derived from partially overlapping regions of the viral genome, with the exception of ORF4 and ORF5 of IAF-exp91 which are separated by nine nucleotides (Conzelmann *et al.*, 1993; Mardassi *et al.*, 1995; Meulenberg *et al.*, 1993b; Murtaugh *et al.*, 1995). The size of each ORF and the deduced number of aa residues encoded by the above mentioned PRRSV strains are indicated in the Table 1.2.

Table 1.2: Comparative ORF lengths of prototype PRRSV strains.

ORF	LV*	PRRS-10†	IAF-exp91‡	ATCC VR-2332 §
ORF1a	7,188	ND	ND	ND
ORF1b	4,389	ND	ND	ND
ORF2	747	747	ND	768
ORF3	795	795	762	762
ORF4	549	549	534	534
ORF5	606	606	600	600
ORF6	519	519	522	522
ORF7	384	384	369	369

* The Netherlands prototype strain (Wensvoort *et al.*, 1991b).

† The german prototype strain (Conzelmann *et al.*, 1993).

‡ The Québec prototype strain (Mardassi *et al.*, 1995)

§ The US prototype strain (Benfield *et al.*, 1992a)

ND: Not determined.

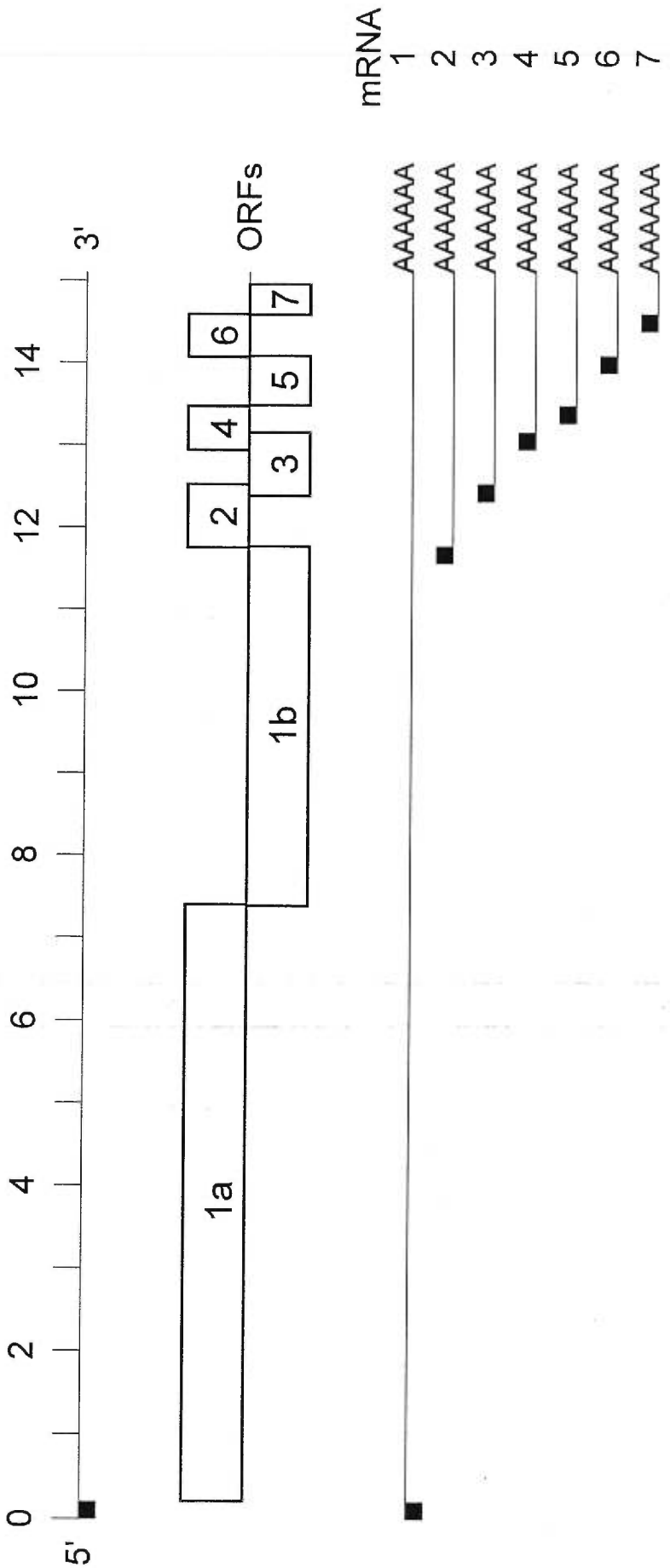

Figure 1.3: Organization of the genome of PRRSV. In the upper part, the ORFs are identified in the nucleotide positions. In the lower part, the 3' nested set of six subgenomic mRNAs are shown. The leader sequence is indicated by a solid square.

2.6 Structural proteins and their nomenclature

Since early isolation of PRRSV strains LV in Europe and ATCC VR-2332 in the US, the presence of 3 major structural proteins with an apparent molecular weights of 25, 19 and 15 kDa in cell lysates of PRRSV-infected cells were reported (Meulenberg *et al.*, 1993b; Nelson *et al.*, 1993). These observations were later confirmed with the Canadian isolates of PRRSV, IAF-exp91 and IAF-Klop (Mardassi *et al.*, 1994a; Mardassi *et al.*, 1995). Gene-specific anti-peptide sera raised in rabbits revealed that the above mentioned proteins are encoded by ORFs 5, 6 and 7, respectively and they are referred to as the major structural protein of PRRSV. Recent findings on structural proteins of LV indicate that translation products of ORFs 2-4 are also incorporated into the viral particle. The association of the latter proteins with the North American isolates of PRRSV remains to be established, taken into consideration the important genomic variations that exist between PRRSV isolated from infected pigs in the two continents.

It was suggested in the "International PRRS Conference" held in Copenhagen in August 1995, to use N for the nucleocapsid protein encoded by the ORF7 and M for the nonglycosylated membrane protein encoded by ORF6. The other four structural proteins of PRRSV (LV strain) encoded by ORFs 2-5 are glycoproteins and they are referred to as GP_2, GP_3, GP_4 and GP_5, respectively (Meulenberg *et al.*, 1997b). Some authors may also use the term "major envelope glycoprotein (E)" for ORF5-encoded glycoprotein, however the nomenclature of Copenhagen PRRS Conference will be adapted to describe the structural proteins of PRRSV in this thesis. Furthermore, we will describe these proteins under two separate headings of major structural proteins of PRRSV comprising the 3 proteins GP_5, M and N, and minor structural proteins of PRRSV which includes GP_2, GP_3 and GP_4 , since the former are rather more abundantly present in the virion. Apart from the LV strain, little is still published relating to the minor structural proteins of the North American strains of PRRSV and our literature review will therefore be

relevant to the findings related to the European isolates of PRRSV. The major characteristics of the structural proteins of both European and North American isolates described in the literature is summarized in Table 1.3.

2.6.1 Major structural proteins

2.6.1.1 GP_5

As for other arteriviruses, the ORF5 of PRRSV encodes a 24.5-26 kDa envelope protein with characteristic hydropathy profile and putative glycosylation sites (Meulenberg *et al.*, 1993b). The Québec reference strain IAF-Klop possesses three potential glycosylation sites at amino acid (aa) positions 33-31, 44-46 and 51-53, shared by the US isolate ATCC VR-2332 (Mardassi *et al.*, 1995; Murtaugh *et al.*, 1995). The GP_5 sequence of LV shows only 2 potential glycosylation sites at aa positions 44-46 and 51-53. It is not clear at present, which of the potential glycosylation sites are occupied by oligosaccharides residues, but it has been demonstrated that the GP_5 incorporated into virion contains complex N-linked oligosaccharides, since it was proven to be sensitive to endoglycosidase F (PNGaseF) digestion and partially sensitive to endo-β-*N*-acetylglucosaminidase H (EndoH) digestion (Mardassi *et al.*, 1996; Meulenberg *et al.*, 1995). It was also suggested that unlike the ORF5 encoded G_L protein of EAV (de Vries *et al.*, 1992), the GP_5 of LV does not contain *N*-acetylgalactosamine, since it is totally resistant to endo-β-galactosidase. In spite of important genomic variations between the North American isolates and LV, the hydropathy profiles of their ORF5 encoded proteins are remarkably similar (Mardassi *et al.*, 1995; Murtaugh *et al.*, 1995). All strains studied so-far possess two putative membrane spanning motifs situated between aa residues 65-130 and 170-190. As in the case of EAV (de Vries *et al.*, 1995), the GP_5 of PRRSV is associated to form hetero-dimers by disulfide links with M protein, since monospecific anti-GP_5 or anti-M sera precipitate both proteins.

Table 1.3. Comparative characteristics of structural proteins encoded by ORF2 to ORF7 of PRRSV

Coding area & gene product		No. of Amino acids			Predicted M_r (kD)			Apparent M_r (kD) on SDS-PAGE			Potential glycosylation sites[¶]		
ORF	Protein	IAF exp-91[†]	VR-2332[‡]	LV[*]	IAF exp-91	VR-2332	LV	IAF exp-91	VR-2332	LV	IAF exp-91	VR-2332	LV
2	GP_2	NA	256	249	NA	29.4	28.4	NA	NA	29-30	NA	2	2
3	GP_3	254	254	256	29.0	29.0	30.6	NA	NA	45-50	7	7	7
4	GP_4	178	178	183	19.6	19.5	20.0	NA	NA	31-35	4	4	4
5	GP_5	200	200	201	22.4	22.4	22.4	24.5	26	25	3	3	3
6	M[§]	174	174	173	19.1	19.0	18.9	19	19	18	1	1	2
7	N[§]	123	123	128	13.6	13.5	13.8	15	15	15	1	1	1

[†] The Quebec reference strain (Mardassi *et al.*, 1995)

[‡] The US prototype strain (Benfield *et al.*, 1995)

[*] The European prototype strain (Wensvoort *et al.*, 1991)

[¶] These numbers indicate putative glycosylation sites. The number of effectively glycosylated sites for each protein is not known.

[§] In spite of potential glycosylation sites, these proteins are not glycosylated.

NA: Not available.

2.6.1.2 M protein

The ORF6 encoded M protein has an estimated M_r of 18.9-19 kDa and shows three highly hydrophobic regions in it's N-terminal half that are assumed to be membrane spanning domains. The M protein of all above mentioned three reference strains also contains one putative N-glycosylation site (Mardassi *et al.*, 1995; Meulenberg *et al.*, 1993b; Murtaugh *et al.*, 1995). The latter appears not to be linked to oligosaccharides since endoglycosidase digestion of the *in vitro* translation product of ORF6 as well as the native viral protein does not result in reduction of molecular weight of the M protein (Mardassi *et al.*, 1996; Meulenberg *et al.*, 1995)

2.6.1.3 N protein

As for EAV and LDV (de Vries *et al.*, 1992; Godeney *et al.*, 1990), the ORF7 of PRRSV encodes the nucleocapsid protein with an estimated M_r of 13.5-13.8 kDa. The N protein of the North American isolates are five aa smaller than LV. Nevertheless, the N-terminal of the protein in all isolates contain basic residues that may facilitate interaction of the nucleocapsid protein with the RNA genome. The N protein also contains one putative N-glycosylation site, which for the same reasons mentioned for the M protein, does not appear to be occupied by carbohydrate residues.

2.6.2 Minor structural proteins of PRRSV

2.6.2.1 GP_2

The ORF2 encoded glycoprotein of PRRSV is the G_s counterpart of EAV, but since it's relative molecular size (29-30 kDa) is greater than the GP_5 (the G_L counterpart of EAV) the nomenclature used for EAV would be inappropriate. The

GP_2 of both LV and ATCC VR-2332 strains contain 2 distinctive hydrophobic peaks. Each peak is formed by approximately 50 aa situated at their carboxyterminals. It includes two putative glycosylation sites which are shared by prototype strains of both continents. Using anti-GP_2 specific anti-peptide or anti-PRRSV pig convalescent sera, it has been established that the GP_2 of LV is incorporated into the extracellular virion (Meulenberg *et al.*, 1996). Compared to the N and M proteins, the estimated quantity of incorporated GP_2 protein in the extracellular virion is apparently smaller favoring the concept that the 29-30 kDa protein encoded by the ORF2 of LV is a minor structural protein. The SDS-PAGE migration pattern of this protein in presence and absence of reducing agent β mercaptoethanol, or the alkylating agents iodoacetamine or *N*-ethylmaleimide, suggests that a fraction of the GP_2 is folded on itself via disulfide bonds, without forming homo- or hetero-multimers with other viral proteins. Finally, the reduction of apparent M_r in gel electrophoresis of GP_2 following endoglycosidase F (PNGaseF) treatment of extracellular virion and it's resistance to endo-β-*N*-acetylglucosaminidase H (EndoH) digestion indicate that the putative *N*-glycosylation site(s) of GP_2 are occupied by complex *N*-glycans.

2.6.2.2 GP_3

With approximately 60% aa identity between the North American and the European isolates, the ORF3 encoded protein is regarded as the least conserved gene of PRRSV (Meulenberg *et al.*, 1997b). The ORF3 of two North American prototype strains reveal a carboxy-terminal deletion of 12 aa compared to LV (Mardassi *et al.*, 1995). This deletion results in a remarkable hydrophilic region associated with the GP_3 of LV whereas the predicted product of North American isolates reveal an amphipathic pattern in this region (Murtaugh *et al.*, 1995). The 12 aa deletion is reflected in difference of estimated M_r of the isolates from two continents (29 and 30.6 for the North American and LV, respectively). As shown in table 1.3, the predicted M_r of GP_3 is remarkably smaller than it's apparent M_r

estimated by gel electrophoresis (Meulenberg *et al.*, 1995). This is explained by the presence of seven N-glycosylation sites (six sites for the Québec strain IAF-exp91) on ORF3 of LV and ATCC VR-2332. The reactivity of MAbs specific to GP_3 of LV revealed that the GP_3 of LV is a structural protein. Endoglycosidase digestion of the recombinant protein expressed in insect cells results in size reduction of the protein and appearance of a band on SDS-PAGE equal to the predicted M_r of the protein (van Neiuwstadt *et al.*, 1996). No counterpart has yet been defined for an ORF3 encoded structural protein amongst other arteriviruses. On the contrary, it has been proposed that the protein encoded by ORF3 of LDV is probably produced in soluble form and not incorporated in the viral particle (Faaberg *et al.*, 1997). These findings are controversial since it is suggested that LDV is the closest to PRRSV amongst all arteriviruses.

2.6.2.3 GP_4

The ORF4 encoded protein has a predicted 19.5-20.0 kDa and contains highly hydrophobic sequences situated at it's amino- and carboxy-terminal regions. The aa sequence of the amino terminal is suggestive of a putative signal sequence. GP_4 also contains four N-linked glycosylation sites which are conserved amongst the European and the North American strains of PRRSV. It was demonstrated that the GP_4 of LV is associated with neutralizing epitopes (van Neiuwstadt *et al.*, 1996) which are not conserved amongst the North American strains and LV, since MAbs specific to the GP_4 of LV could neutralize the homologous strain *in vitro*, but not the US prototype strain ATCC VR-2332. The neutralizing domain has been mapped to a hydrophilic, exposed region adjacent to amino terminal region of GP_4 (aa position 59-67) which appears to be highly variable amongst the European strains (Meulenberg *et al.*, 1997a). Furthermore, it was established that the GP_4 contains complex type *N*-glycans acquired during the transport of the protein through endoplasmic reticulum and Golgi compartment. It is of interest to note that the GP_4 is not detected by convalescent pig sera (Meulenberg *et al.*, 1995).

The ORF4 gene of a North American strain of PRRSV has also been cloned and expressed in *E. coli* (Kwang *et al.*, 1994). Only 65% of PRRSV-positive sera obtained from affected pig farms reacted positively by immunoblotting with the recombinant ORF4 protein. Therefore the implication of ORF4 encoded protein in inducing neutralizing antibodies following PRRSV infection is yet to be determined.

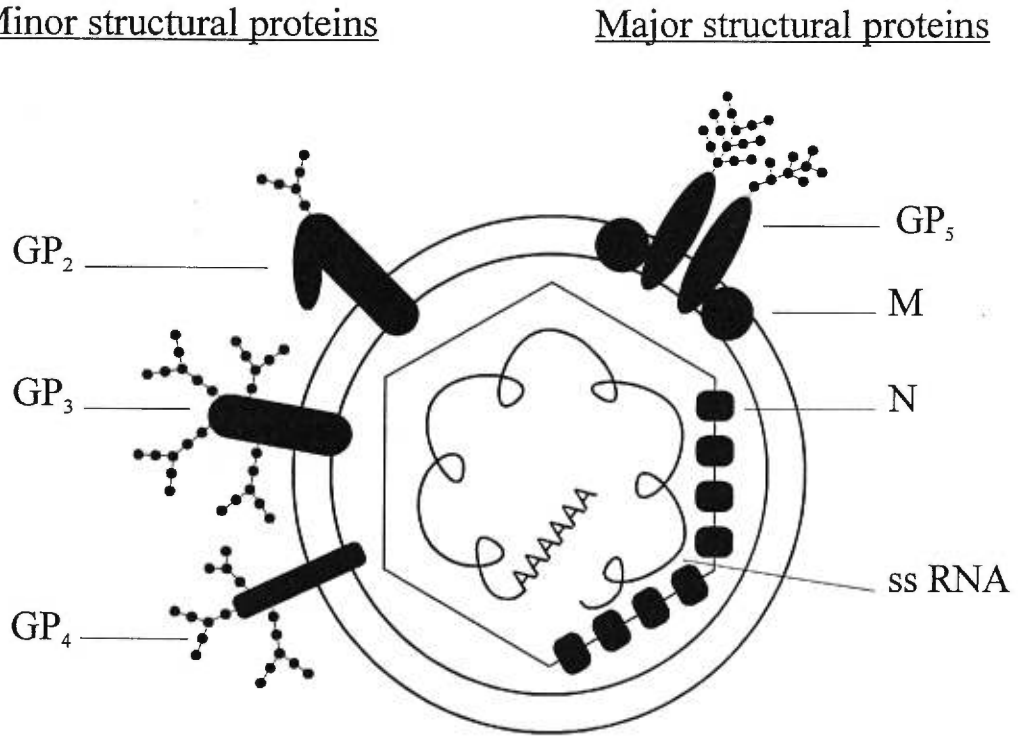

Figure 1.4 Schematic presentation of PRRS virus (LV strain)

2.7 Genomic variability of PRRSV

Comparison of the aa sequences encoded by the ORFs of different PRRSV isolates with those of other viruses belonging to the order of *Nidovirales* indicate that PRRSV is distantly related to the coronaviruses and closely related to LDV and EAV (Conzelmann *et al.*, 1993; Meulenberg *et al.*, 1993). The aa sequences of ORF1a and ORF1b contain elements conserved in the RNA polymerase of Berne virus (BEV) (Snijder *et al.*, 1990) and coronaviruses (Boursnell *et al.*, 1987; Briefly *et al.*, 1987; Lee *et al.*, 1991). Nucleotide sequence variability associated with ORF1b of the North American and the European isolates allowed typing these two genotypes by multiplex PCR of the RNA directly extracted from the supernatant of PRRSV-infected cell cultures (Gilbert *et al.*, 1997). As in the case of other RNA viruses, a high degree of genomic variability has been reported for PRRSV (Kapur *et al.*, 1996). However, based on degree of genomic identity, the North American and the European isolates can be separately regrouped in two distinct genotypes. Field isolates of each continent have a greater degree of relatedness to each other than the strains originated from the other continent.

The high degree of genomic distance between the isolates from the two continents have persuaded some experts to hypothesize that the European and the North American PRRSV are in fact two distinct viral species that have originated from a common ancestor. Such assumption is unlikely, taking into consideration the identical biochemical and morphologogical characteristics, the similarities in genomic organization and replication strategies, the type of disease caused in pigs and identical permissive cells. Several North American and European strains have been partially sequenced and so far, LV remains the only PRRSV to be completely sequenced. Percentage of aa identity of structural and non-structural proteins of LV strain compared to other reference strains of PRRSV and other *Arterivirus* are shown in Figure 1.2 and Table 1.4. As can be seen, PRRSV is more related to LDV than to EAV, and ORF3 and ORF5 are the most variable regions of PRRSV.

2.7.1 Variability of ORFs encoding major structural proteins

The GP_5 can be regarded as the least conserved protein of PRRSV. Most of the aa substitutions are clustered in a hypervariable region adjacent to the amino-terminal signal sequence which also involves N-linked glycosylation sites, varying from two to three (Andreyev *et al.*, 1997, Meng *et al.*, 1995a). This may explain in part, the difference in apparent M_r on SDS-polyacrylamide gels of the GP_5 of PRRSV isolates (Magar *et al.*, 1997). A similar degree of aa variability (~88% identity) exists for the GP_5 of the European strains (Suárez *et al.*, 1996).

The M protein of PRRSV is relatively well conserved with greater than 96% aa identity between the North American or European PRRSV isolates. Antigenic variability can be detected between North American and European strains by MAbs specific to the M protein (Dea *et al.*, 1996). Sequencing data revealed greater than 78% aa identity between the North American and the European strains (Table 3). Antigenic variability amongst the North American strains can also be detected by MAbs specific to the M protein (Dea *et al.*, 1996).

The N protein of PRRSV is also encoded by a relatively well conserved region of the viral genome since a high degree of aa sequence identity has been observed amongst the N protein of North American (96-100%) and European (94-99%) strains (Meng *et al.*, 1995b; Suárez *et al.*, 1996b). As expected, the aa sequences of the N protein of North American isolates displayed only 67-81% identity with European strains. MAbs specific to the N protein recognized epitopes specific to or shared by the North American and European isolates (Dea *et al.*, 1996; Drew *et al.*, 1995 and 1997; Nelson *et al.*, 1993). A highly conserved region consisting of 54 aa residues, shared by the North American and the European isolates was mapped to the middle third (aa position 47-93) of the N protein (Rodriguez *et al.*, 1997).

Table 1.4: Percentage amino acid sequence identity of ORFs 2 to 7 of LV with those of other PRRSV strains and arteriviruses of other animal species

LV[a]	PRRSV-10[b]	VR-2332[c]	VR-2385[d]	IAFexp-91[e]	N. American strains[f]	LDV-P[g]	LDV-C[h]	EAV[i]
ORF2	99	63	62	ND	91-99	38	32	NS
ORF3	99	60	57	54	86-98	31	28	NS
ORF4	99	70	69	68	92-99	29	30	NS
ORF5	99	55	54	52	88-97*	47	47	NS
ORF6	100	79	78	81	96-100*	53	53	23
ORF7	100	64	57	59	96-100*	41	44	20

[a] Meulenberg *et al.*, 1993b, Meulenberg *et al.*, 1997b

[b] Conzelman *et al.*, 1993.

[c] Murtaugh *et al.*, 1995.

[d] Meng *et al.*, 1994.

[e] Mardassi *et al.*, 1995.

[f] Comparison amongst the North American strains, Meng *et al.*, 1995a, Meng *et al.*, 1995b, Andreyev *et al.*, 1997.

[g] Chen *et al.*, 1993.

[h] Godeney *et al.*, 1993;

[i] den Boon *et al.*, 1991;

* Including the Canadian isolate IAF-exp91.

ND: not determined.

NS: not significant.

This fragment defines a continuous antigenic domain recognized by MAbs which react with both homologous Spanish strain and a North American PRRSV isolate. Other antigenic domains reported in the above study are discontinuous epitopes that mapped to unidentified region(s) of the N protein. The non-coding 3' terminal region of North American isolates of PRRSV exhibits 59% nucleotide identity with that of European isolates and has been found to be a useful tool to differentiate the North American from European isolates by multiplex RT-PCR (Mardassi *et al.*, 1994b) or by *in situ* hybridization (Larochelle *et al.*, 1996) experiments.

2.7.2 Genomic variability of ORFs encoding minor structural proteins

The degree of aa identity amongst the US PRRSV isolates varied from 91% to 99% for ORF2 and 86% to 98% for ORF3 (Katz *et al.*, 1995; Meng *et al.*, 1995a, Morozov *et al.*, 1995). According to a recent report, the ORF3 product of the UK PRRSV isolates have a hydrophilic hypervariable region proximate to the carboxy-terminal region that overlaps with ORF4, resulting similarly in a hypervariable region situated at the amino-terminal extremity of the GP_4 (Drew *et al.*, 1997). This region of the GP_4 is associated with neutralizing domains of LV (Meulenberg *et al.*, 1997a). A motif consisting of nine aa (positions 59-67) forms the core of the neutralizing domain and isolates with aa deletion or substitution(s) in these positions fail to react with the panel of MAbs specific to GP_4 of LV. A relatively high degree of aa identity (92-99%) has been reported for the GP_4 of the US PRRSV isolates (Meng *et al.*, 1995a).

3. Pathogenesis of PRRSV infection and characteristics of immune response

3.1 Viral Pathogenesis

PRRSV replicates extensively in the lungs and virus titers as high as $10^{5.5}$to $10^{5.9}$ $TCID_{50}$/g of lung tissue can be obtained (Van Reeth, 1997). The virus has a very restricted tropism for macrophages of the alveolar spaces and alveolar septa (Halbur *et al.*, 1994; Pol *et al.*, 1991). Primary cultures of porcine alveolar macrophages (PAM), however, make up 80-90% of the infected lung population (Duan *et al.*, 1997) and these cells undergo rapid cytopathic effects. Gross pneumonitis with lobular red areas has been reported in some experimental inoculation studies (Collins *et al* 1992b; Halbur *et al* 1993; Halbur *et al* 1994; Halbur *et al* 1995; Paton *et al* 1992; Pol *et al* 1991; Rossow *et al* 1995). In microscopic examination, there is a marked thickening of alveolar septa containing many macrophages, fewer lymphocytes and type II pneumocyte proliferation. Alveolar lumina are filled with inflammatory cells and necrotic cell debris. While cells collected from lung lavages of uninfected control pigs contains greater than 95% macrophages, lavage fluids from PRRSV-infected pigs at the seventh PID contain about 50% macrophages, 35% neutrophils and 15% lymphocytes (Zhou *et al,* 1992).

The tropism of PRRSV for PAMs has led to the hypothesis that lung defense is suppressed as a result of PRRSV infection but this hypothesis is debatable for the following reasons:

a- at any PI period, PRRSV cannot be detected in more than 1% of PAMs, but this low proportion of PRRSV antigen detected in culture of infected cells may also be an artefact since infected cells lose their adherence capacity to plastic

surface and are discarded with wash buffers during test procedure (Mengeling *et al,* 1995).

b- a similar proportional reduction of PAMs has been observed in other viral infections of the lung such as porcine respiratory coronavirus and swine influenza, largely reflecting the chemotaxis of neutrophils and lymphocytes into the alveolar spaces as a result of infection.

c- there is insufficient evidence that PAM's functions in PRRSV-infected lungs are seriously hampered. In one experiment (Zhou *et al.,* 1992) at 1 week PI, PAMs from PRRSV-infected pigs were impaired in their capacity to synthesize peroxide anions, but expression of interleukine-1β (IL-1β) was enhanced.

3.2 Secondary infections associated with PRRS

Field observations suggest an increase in atrophic rhinitis, polyserositis and bacterial meningitis in PRRSV-infected pigs (Collins 1991a). PRRSV infection may predispose pigs to secondary infections by damaging non-specific respiratory defense through the destruction of alveolar macrophages that may be substituted by immature cells (Austin & Bistrom, 1991; Molitor, 1993) and by inducing inflammation in the nasal mucosa (Collins *et al.,* 1992b; Galina 1995; Rossow *et al.,* 1995). In one study, experimentally PRRSV-infected pigs were found to be predisposed to *Streptococcus suis* (Galina *et al.,* a and b). Infection of susceptible pigs with PRRSV, followed by intra-nasal challenge with *S. suis* resulted in central nervous clinical signs and meningitis. Other workers have suggested interaction between PRRSV and *Actinobacillus pleuropneumoniae,* resulting in aggravated clinical signs and lesions (Wensvoort, 1995). Pigs co-infected with porcine respiratory coronavirus (PRCV) and PRRSV developed more severe clinical signs (Van Reeth *et al.,* 1994). No conclusive results in terms of interaction between PRRSV and bacterial pathogens could be obtained from infection of pigs with PRRSV and a subsequent challenge with *Pasteurella multocida* (Carvalho *et al.,*

1997). Infection of susceptible pigs with PRRSV followed by intra-tracheal challenge by *Haemophilus parasuis* did not exacerbate polyserositis lesions compared with the group where *H. parasuis* was the unique challenge (Solano *et al.*, 1997). Experimental studies indicate that concomitant infection of PRRSV with influenza H3N2 virus or PRRSV with *A. pleuropneumoniae* did not enhance the severity of secondary infections of the respiratory tract (Pol *et al.*, 1997). According to a two-phase study (Cooper *et al.*, 1995), PRRSV infection did not potentiate pathogenesis of bacterial challenge with *H. parasuis, P. multocida, S. suis, Salmonella choleraesuis* and *A. pleuropneumoniae*. *Mycoplasma hyopneumoniae* is one of the pathogens commonly found in concomittant infection with PRRSV in the affected farms (Zeman *et al.*, 1993). Experimental findings suggest that PRRSV infection does not exacerbate the severity of *M. hyopneumoniae* in young pigs (Van Alstine *et al.*, 1996). This observation is in agreement with a previous report (Albina *et al.*, 1995b), indicating that PRRSV infection had no significant effect in predisposing pigs to pseudorabies virus (PRV) or *M. hyopneumoniae*. In contrast to previously published field observations (Done & Paton, 1995) where endemic PRRSV was reported to produce an increased occurrence of secondary respiratory infections, controlled experimental infection results did not confirm the role of PRRSV in potentiating bacterial diseases. Mismanagement factors such as introducing pigs to the herd from different sources, herd health, bad housing conditions, over-crowding, temperature variations, weaning stress and nutrition short comings including insufficient feeder and waterer length may all contribute to weakening the immune function and enhance disease outbreaks.

3.3 Effect of PRRSV on the immune system

PRRSV was first isolated on PAMs (Pol *et al.*, 1991, Wensvoort *et al.*, 1991b) and so far they remain the only porcine cells used for tissue culture propagation of PRRSV. Yet, generalized viremia is detected in PRRS and the virus persists in, or it is shed from oropharyngeal tissues of the upper respiratory tract (Wills *et al.*, 1997b), semen (Christopher-Hennnings *et al.*, 1995a; Swenson *et al.*, 1994), muscle, spleen and the accessory lymphatic nodes (Christopher-Hennnings *et al.*, 1995b; Yaeger *et al.*, 1993). Therefore, replication in PAMs alone does not fully explain the generalized aspect of PRRSV infection. It was demonstrated that other immune system cell populations such as spleen macrophages, brain microglia and M-CSF induced adherent peripheral blood monocytes are highly permissive to PRRSV, producing titres of 10^4-10^7 $TCID_{50}$/ml (Molitor *et al.*, 1997, Voicu *et al.*, 1994). T and B lymphocytes have also been found permissive to PRRSV, producing only low progeny titres of infective virus (10-10^2 $TCID_{50}$/ml)(Molitor *et al.*, 1997). Furthermore, it was suggested that *ex vivo* expression of ORF5 encoded protein of PRRSV may induce apoptosis in COS7 cells and PAMs (Suárez *et al.*, 1996). Should PRRSV replication exert the same effect on all of the above mentioned host permissive cells, a profound immunosuppressive effect should be associated with PRRSV infection. These findings suggest that PRRSV may play a possible role in immuno-suppression and enhancement of secondary infections. As was previously mentioned, this is a highly controversial subject. Reports based on clinical observations would favor such a role (Collins & Rossow, 1993; Done & Paton 1995; Galina *et al.*, 1994b; Van Reeth *et al.*, 1994; Zemzan *et al.*, 1993) whereas the results of experimental studies are either inconclusive or clearly indicate that PRRSV has no significant role in exacerbation of secondary infections (Albina *et al.*, 1995a and b; Carvalho *et al.*, 1997; Cooper *et al.*, 1995; Pol *et al.*, 1997; Solano *et al.*, 1997; Van Alstine *et al.*, 1996). A review of published data relating to the changes occurring in immune parameters of pigs after exposure to virus may help clarify the possible immuno-suppressive role of PRRSV.

3.4 Effect of PRRSV on functional parameters of immune cells

Cellular populations and functional parameters of the immune system from PRRSV-infected pigs have been studied in some details (Molitor *et al.*, 1997; Nielsen & Bøtner 1997; Shimizu *et al.*, 1996; Vézina *et al.*, 1996). Naturally PRRSV-infected pigs (7-9 weeks old) showed no changes in total number of peripheral blood lymphocytes, whereas a significant reduction of total mature peripheral T lymphocytes ($CD2^+$), helper T cells ($CD4^+$) and the $CD4^+/CD8^+$ ratio was observed (Shimizu *et al.*, 1996). These changes could not uniquely be attributed to PRRSV infection, since no information related to other pathogens which may have infected animals under observation is available. In experimental PRRSV infection trials, eight-week-old SPF pigs showed a transient decrease in $CD2^+$ and $CD4^+$ cells between the third and PID 14, but the $CD4^+/CD8^+$ ratio consistently remained below pre-inoculation levels during four weeks. These results could not be reproduced with hysterectomy-delivered, colostrum-deprived pigs. The above transient reduction in number of mature thymocytes and persistent decrease of $CD4^+/CD8^+$ ratio has also been reported by an independent investigator (Nielsen & Bøtner, 1997). The temporary drop in peripheral blood lymphocytes and their rapid return to normal levels may be associated with intense lymphocyte infiltration of the infected lungs, rapidly compensated by generation of new cells. This view is supported by the fact that lymphocytic and neutrophilic counts rise proportionally in the broncho-alveolar lavage fluids of PRRSV-infected pigs (Molitor *et al.*, 1997). The number and function of natural killer cells (NK) remain unaltered following infection (Albina *et al.*, 1995b). The mitogen-induced lympho-proliferative response of the PRRSV-exposed animals may remain normal (Bautista & Molitor, 1997) or show a transient decline at the third PID (Vézina *et al.*, 1996). Interestingly, this decline is coincident with the period of temporary drop in peripheral $CD2^+$ cells. A significant *ex vivo* spontaneous blastogenesis was recorded in lymphocytes obtained from PRRSV-infected pigs between 7-21 PID which may be associated with the altered cytokine secretion pattern of immuno-

competent cells. In conclusion, the only consistent variation remarked in immune parameters of PRRSV-infected animals is a drop in $CD4^+/CD8^+$ but it is not clear if this reduction is sufficient to induce an immunosuppression. Mammals in general do not manifest a severe immunodeficiency to develop secondary infections following minor reductions in T helper population.

3.5 Cell mediated immunity to PRRSV

Intracellular pathogens such as viruses are usually effectively cleared by cell mediated immune response, which involves antigen specific cytotoxic and helper T cell effector populations. In one study, $SLA^{c/c}$ minipigs were used to evaluate the cellular immune response of PRRSV-infected animals (Bautista & Molitor, 1997). Peripheral blood mononuclear cells (PBMC) obtained from PRRSV-infected animals and incubated *ex vivo* in presence of viral antigen underwent blastogenic transformation. Blastogenesis blocking test with MAbs specific to porcine T and B subpopulations defined $CD4^+$ T cells as PRRSV antigen specific effector cells. Re-exposure of the animals to virus had a booster effect on antigen induced *ex vivo* blastogenesis. Cell mediated immunity was confirmed by detecting delayed-type hypersensitivity (DTH) to viral antigen (Bautista & Molitor, 1997). These results indicate that in spite of carrier state, PRRSV- infected pigs remain immuno-competent and can effectively mount a cellular immune response. But it is not clear if the immune condition of the infected animals is capable to eliminate virus and resist re-infection.

3.6 Antibody response to PRRSV infection

Antibodies specific to the three major structural proteins of PRRSV (GP_5, M and N) are detectable by Western immunoblotting (WB) in sera of naturally or experimentally PRRSV-exposed pigs (Loemba *et al.*, 1996; Mardassi *et al.*, 1995; Meulenberg *et al.*, 1995). Levels of PRRSV-specific antibodies detectable by

indirect immunofluorescent assay (IIF) (Yoon *et al.*, 1992b) appear in sera as early as sixth PID and persist for at least nine weeks (Loemba *et al.*, 1996; Joo *et al.*, 1997). IgM antibodies are first detected on PID six to ten, and after reaching maximum titers by the PID 28, titers gradually diminish to undetectable levels by the PID 42 to 63. Appearance of IgG antibodies is delayed by almost 10 days (Loemba *et al.*, 1996), or these are first detected simultaneously with IgM (Joo *et al.*, 1997). Neutralizing antibodies at titers greater than 16 do not appear until PID 28 to 35 (Loemba *et al.*, 1996; Yoon *et al.*, 1995). The results obtained by these authors indicate that irrespective of the detection method (ELISA, IPMA or IIF, Yoon *et al.*, 1995), the highest antibody titers are detectable around three weeks post-infections and they persist for 100 days and more. While high titers of IgM antibodies indicate a recent exposure to PRRSV, development of neutralizing antibodies are delayed to around four weeks post-exposure.

3.7 Antibody dependent enhancement (ADE)

Antibodies may enhance virus infection of Fc receptor positive cells through the process of viral opsonization by antibodies after formation of immune complexes (Peiris *et al.*, 1981), as it was demonstrated that anti-Fc receptor MAbs blocked ADE of viral replication in macrophages. This phenomenon has been described for a number of viruses including Dengue viruses (Halstead *et al.*, 1977), feline infectious peritonitis virus, (Olsen *et al.*, 1992), West Nile virus (Peiris *et al.*, 1981) and PRRSV (Choi *et al.*, 1992). Following passive immunization of pigs with anti-PRRSV specific IgG, mean level and duration of viremia is substantially greater in pigs having sub-neutralizing levels of anti-PRRSV IgG compared to the pigs injected with identical quantities of unrelated IgG. In contrast, virus replication is significantly inhibited in pigs with neutralizing antibody levels (Yoon *et al.*, 1996). ADE phenomenon can be detected in porcine sera between PID 20 to 62 and it may contribute to increase the susceptibility to PRRSV infection of young pigs during a period when maternal antibodies are declining (Zemen *et al.*,

1993). ADE may also be a contributing factor to post-vaccination exacerbation of PRRS reported from some herds (Yoon *et al.*, 1997). Genomic and antigenic variability of PRRSV result in failure of the vaccine to induce sufficient levels of neutralizing antibodies against pathogenic strains to which the animals will later be exposed. This view is supported by a recent experiment (Yoon *et al.*, 1997), where virus yields were significantly reduced, when the autologous strain and four of the 16 field isolates were incubated on PAMs in presence of neutralizing concentrations of a porcine serum specific to the autologous strain. Preincubation of all strains with subneutralizing levels of the same serum significantly increased the virus yields. Subneutralizing levels of serum antibodies enhanced replication of the homologous virus strain, proportionally to the serum dilution. No correlation was found between serum neutralization capacity or antibody concentration and the ADE phenomenon for heterologous strains, suggesting that independent antigenic determinants are implicated in VN and ADE activity.

3.8 Immunization against PRRSV

Since the appearance of PRRS almost a decade ago, hundreds of scientific papers have been published dealing with various aspects of pathogenesis, epidemiology, molecular and structural characteristics of the virus. However, in spite of remarkable scientific progress and the important economical impact of the disease, little progress has been made in relation with a practical immunization strategy to protect the swine industry against heavy economical losses resulting from PRRS outbreaks. PRRSV has several characteristics that can hinder development of an effective vaccine:

1- The genomic variability of PRRSV interferes with designation of any single strain for use in attenuated vaccine. As was previously described, the neutralizing domains of the GP_4 protein are situated in the hypervariable area of the protein (Meulenberg *et al.*, 1997a). The GP_5 protein which is known to be associated with neutralizing epitopes in other arteriviruses (Balasuriya *et al.*,

1993; Balasuriya *et al.*, 1995a; Coutelier & Van Snick, 1988) is the least conserved genomic area of PRRSV. Other major structural proteins (M and N), which are more conserved, are not associated with neutralizing epitopes (Dea *et al.*, 1996). Therefore the antibodies directed against these proteins may even exacerbate infection in vaccinated animals through the ADE phenomenon.

2- PRRS is a persistent infection, therefore inoculation of an attenuated strains may result in virus persistence, shedding of new mutants and eventually disease outbreaks due to propagation of such mutants (Wills *et al.*, 1997a).

3- Stress related immuno-suppression which is a common problem in intensive animal production, may potentiate virulence of persistent attenuated virus and result in post-immunization disease outbreaks.

4- Contrary to what has been proposed (Plana Duran *et al.*, 1997), inactivated vaccines are probably inappropriate specially in breeding farms where animals are reared for longer periods and a durable immunity is therefore desirable. Generally, immunization by inactivated vaccines results in short term protection due to a weak cellular immune response (Molitor 1993; Molitor *et al.*, 1997). Furthermore, ADE may contribute to development of severe clinical disease. Low yields of PRRSV progeny in tissue culture (Benfield *et al.*, 1992a, Collins *et al.*, 1992a) renders the production costs too high and the final product will be economically unfeasible.

While the important role of cellular immune response in eliminating viral aggression has been well established, little data relating to the mechanism of cellular immune response following PRRSV infection is available at this time. A subunit recombinant type vaccine consisting of conserved neutralizing domains of PRRSV may be the ideal product for field application but based on our actual knowledge of structural proteins of PRRSV, such a product may not be available in the near future.

Presently, a commercial live-attenuated vaccine (RespPRRS-NOBL Laboratories/Boehringer Ingelheim) is approved in pigs from three to eighteen weeks of age, excluding the breeding farms. Strangely enough, no published data is available in relation with the safety and/or efficacy of this product. According to unofficial sources, some post-immunization outbreaks PRRS are attributed to this vaccine.

4. Diagnosis of PRRS

4.1 Detection of PRRSV

Virus isolation: Final confirmation of PRRSV infection may depend on virus isolation from affected animals as described by several authors (Benfield *et al.*, 1992b; Dea *et al.*, 1992b; Wensvoort *et al.*, 1991b). PRRSV has been isolated from serum or tissue homogenates of various organs such as spleen, liver, kidney and lungs of the affected animals or aborted piglets. Some attempts have failed to isolate virus from autolysed fetuses, possibly due to rapid virus inactivation at 37° C (Bøtner *et al.*, 1994; Christianson *et al.*, 1992). Virus can be recovered from experimentally-infected pigs on the third or fourth PID. Some PRRSV strains to be isolated from clinically affected pigs may not initially propagate in cultures of MARC-145 or CL 2621 cells (Yoon *et al.*, 1992a). It is therefore preferable to attempt virus isolation on PAMs obtained from SPF pigs, wherever possible.

4.2 Detection of PRRSV antigen

Immunohistochemical detection of PRRSV in frozen sections or formalin-fixed tissues by the use of immunogold, silver staining or IPMA staining has been described (Larochelle & Magar 1995; Magar *et al.*, 1993; Pol *et al.*, 1991) and

can be used for retrospective detection of infection. Immunohistochemical methods were demonstrated to be of value in detection of PRRSV antigen in CNS tissues (Rossow *et al.*, 1996; Thanawongnuwech *et al.*, 1997). Expression of PRRSV proteins or it's replication can simply be detected by IPMA or IIF (Collins *et al.*, 1992b) on fixed tissue cultures.

4.3 Detection of viral genome

RT-PCR is another tool for the detection of PRRSV and for differentiation of the American strains and European strains (Mardassi *et al.*, 1994c; Gilbert *et al.*, 1997). This may also be a valuable tool for detection of PRRSV with reduced virus infectivity such as semen, or when the virus is completely inactivated in specimens (Suárez *et al.*, 1994, Van Woensel *et al.*, 1994). A multiplex RT-nested PCR (TR-nPCR) has been described to differentiate virus shedding in boar's semen originating from a natural infection or live attenuated vaccination. This method could detect as little as 0.01 $TCID_{50}$ of PRRSV per reaction (Shin *et al.*, 1997).

In situ hybridization by a non-radioactive cDNA probe labelled with digoxigenin and targeting the viral RNA encoding the N protein of PRRSV, was described to detect virus in cell cultures and formalin-fixed paraffin embedded tissue sections (Larochelle *et al.*, 1996). The method was found to be highly sensitive, specific and appropriate for retrospective diagnosis.

4.4 Detection of antibodies

4.4.1 Immunoperoxidase on monolayer assay and indirect immunofluorescence

These two tests were described when PRRSV was first isolated (Wensvoort *et al.*, 1991b; Collins *et al.*, 1992b). Both tests can be performed on PAMs or

MARC-145 cell lines. While IPMA is more used in Europe, the IIF is most commonly used in North America with equal specificity and sensibility (Park *et al.*, 1995; Yoon *et al.*, 1992b). Both tests can detect PRRSV-specific antibodies as early as PID seven to fourteen.

4.4.2 ELISA

An indirect ELISA was described (Albina *et al.*, 1992a) for the detection of PRRSV antibody on PRRSV-infected PAMs. A blocking ELISA, also based on fixing whole viral antigen was described (Houben *et al.*, 1995) and found to possess higher sensitivity and specificity than the tests mentioned above. Presently a commercial ELISA kit is available (IDEXX) for detection of anti-PRRSV antibodies. Recently a comparative study was made on different serological methods (IPMA, IIF and ELISA) using the standard methods for the first two tests and an indirect double sandwich ELISA kit (Cho *et al.*, 1997). The overall performance of the ELISA was superior in terms of sensitivity and specificity.

PART II

METHODOLOGY AND RESULTS

CHAPTER 1

CHARACTERIZATION OF MONOCLONAL ANTIBODIES DIRECTED TO ORF5 ENCODED MAJOR ENVELOPE GLYCOPROTEIN (GP_5) OF PRRS VIRUS

ARTICLE 1

Monoclonal Antibodies to the ORF5 Product of Porcine Reproductive and Respiratory Syndrome Virus Define Linear Neutralizing Determinants

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1.1 Summary

Complementary DNA encoding the ORF5 gene of a Québec reference isolate (IAF-Klop) of porcine reproductive and respiratory syndrome virus (PRRSV) has been cloned into the procaryotic expression vectors pGEX-4T and pET21a to produce ORF5-glutathione-S-transferase and ORF5-polyhistidine fusion proteins. Five hybridoma cell lines producing monoclonal antibodies (MAbs) to the 25-kDa viral envelope glycoprotein (GP_5) were obtained from BALB/c mice immunized with the affinity chromatography purified GST-ORF5 fusion protein. The polypeptide specificity of these anti-PRRSV MAbs, belonging to the IgG1 isotype, was confirmed by Western immunoblotting assays with recombinant and native viral proteins, and by radioimmunoprecipitation (RIPA) using [^{35}S]-methionine-labelled concentrated extracellular virus. All these MAbs showed virus-neutralizing (VN) activity, with VN titres ranging from 1:32 to 1:128. Two MAbs (MAbs IAF-1B8 and IAF-8A8) reacted with similar titres with the modified live attenuated vaccine strain ATCC VR-2332, but all five failed to react to the prototype European strain, the Lelystad virus, in VN and indirect immunofluorescence (IIF) tests. The results obtained suggest that these five anti-PRRSV MAbs are directed to serotype-specific linear neutralizing epitopes which are not affected by the absence of carbohydrate residues.

1.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important viral disease first described in North America in 1987 (Goyal, 1993) and in Europe in 1990 (Paton *et al.*, 1991; Wensvoort *et al.*, 1991a). The disease is characterized by inappetence and severe reproductive failure in sows of any parity including late-term abortions, increased numbers of still-born, mummified and weak-born piglets, and respiratory problems affecting pigs of all ages (Goyal, 1993). The causative agent of PRRS was first isolated in The Netherlands in 1991 and designated Lelystad virus (LV) (Wensvoort *et al.*, 1991b), and then in 1992 in the United States and Canada (Collins *et al.*, 1992b; Dea *et al.*, 1992b). Although the clinical syndromes associated with PRRS virus (PRRSV) infection are similar in North America and Europe (Goyal, 1993), strains from both continents represent two distinct genotypes (Mardassi *et al.*, 1994b; Meng *et al.*, 1995a and 1995 b), and significant antigenic differences have been observed (Dea *et al.*, 1996; Nelson *et al.*, 1993; Wensvoort *et al.*, 1992b).

The PRRSV is a small enveloped RNA virus with a 25 to 30 nm isometric core which is closely related to members of the genus *Arterivirus* in morphology, genome organization, transcription strategy and macrophage tropism (Plagemann *et al.*, 1992). Other members of this group include lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV) and simian haemorrhagic fever virus. The PRRSV genome is a single-stranded polyadelynated RNA molecule of about 15kb in length, being the largest amongst arteriviruses (Conzelmann *et al.*, 1993; Mardassi *et al.*, 1995; Meulenberg *et al.*, 1993b). It contains two large open reading frames (ORFs), ORFs 1a and 1b, situated at the 5' extremity of the genome and 6 smaller ORFs, ORFs 2 to 7, located at the 3' part of the genome which are expressed in the infected cells as a nested set of subgenomic RNAs (Conzelmann *et al.*, 1993). ORF1a and ORF1b, which represent nearly 75% of

the genome, code for proteins with apparent polymerase and replicase activity. Previous studies demonstrated that ORFs 5, 6, and 7 code for the major structural proteins of the virion: a 25-kDa glycosylated envelope protein E or GP_5, an 18 to 19 kDa integral membrane protein M and a predominant 14 to 15 kDa nucleocapsid protein (Meulenberg *et al.*, 1995; Mardassi *et al.*, 1995; Mardassi *et al.*, 1996). Recently, three other structural membrane associated glycoproteins have been described for LV (Van Nieuwstadt *et al.*, 1996; Meulenberg *et al.*, 1996). These include a 29 to 30 kDa GP_2, a 45 to 50 kDa GP_3 and a 31 to 35 kDa GP_4 encoded by ORFs 2, 3 and 4, respectively. The equivalent of these structural proteins for the North American strains of PRRSV are yet to be identified. The role of the GP_5 of PRRSV in relation with virus neutralization (VN) and cellular receptor binding is still to be determined, mainly due to the lack of specific immunological probes, while such functions have been clearly established for the G_L protein encoded by the ORF5 of EAV (Balasuriya *et al.*, 1995a; Chirnside *et al.*, 1995a; Deregt *et al.*, 1994). Monoclonal antibodies (MAbs) to the GP_4 of LV have been reported to possess neutralizing properties against the autologous strain and some but not all of the tissue culture-adapted European strains tested (Van Nieuwstadt *et al.*, 1996).

Apparently the GP_5 of PRRSV has low immunogenic properties since immunizing mice with the entire viral particle has so far resulted in isolation of MAbs directed against other viral structural proteins (Dea *et al.*, 1996; Drew *et al.*, 1995; Nelson *et al.*, 1993; Van Nieuwstadt *et al.*, 1996; Wieczorek-Krohmer *et al.*, 1996). In this study, we describe the characterization of neutralizing MAbs which were obtained to the *Escherichia coli*-expressed ORF5 gene product of a reference tissue culture-adapted Québec strain of PRRSV, and the finding that targeted epitopes are linear and not affected by the absence of glycosylation. We further demonstrated that resulting neutralizing MAbs selectively reacted with the homologous PRRSV isolate and the US vaccine strain ATCC VR-2332, but failed to react with the European prototype LV strain.

1.3 Materials and methods

1.3.1 PRRSV and tissue culture

The Québec reference cytopathic strain IAF-Klop of PRRSV (Mardassi *et al.*, 1994b) was plaque-purified twice and propagated in MARC-145 cells, a clone of MA-104 cells highly permissive to PRRSV (Kim *et al.*, 1993), graciously provided to us by J. Kwang (U.S. Meat Animal Research Center, USDA, Agricultural Research Service, Clay Center, Nebraska). The virus strain yielded titres of 10^5-10^6 $TCID_{50}$/mL after five successive passages in MARC-145 cells.

1.3.2 Production of recombinant PRRSV ORF5 fusion proteins.

Genomic RNA was extracted from concentrated extracellular virions by the one-step guanidinium isothiocyanate-acid phenol method (Sambrook et al., 1989). Then, the entire ORF5 coding sequence of PRRSV was amplified by RT-PCR using the following oligonucleotide primers:
1005PS (sense): 5'GGATCCATGTTGGGGAAATGCTTGACC3';
1005PR (antisense): 5'GGATCCGGCAAAAGTCATCTAGGG3'.

These primers, containing *Bam*HI restriction site at their 5' ends, corresponded to the sequence adjacent to the first ATG or the stop codon of the ORF5 coding regions of the Québec strain IAF-exp91 of PRRSV (EMBL/GeneBank accession number L40898). The resulting cDNA was inserted into procaryotic expression vector pGEX-4T1 (Pharmacia Biotech Inc.), as previously described (Mardassi *et al.*, 1996) to obtain pGEX-5 recombinant plasmid. Alternatively, *Bam*HI and *Xho*I recognition sites were added at the 5' ends of the above sense and antisense PCR primers for directional cloning into procaryotic expression vector pET21a (Qiagen). The resulting recombinant plasmid is referred to as pET21a-5.

Sequencing analysis of the recombinant plasmids pGEX-5 and pET21a-5 demonstrated that no alteration or mutation had occurred as a result of PCR amplification. The pGEX-5 encoded a fusion protein consisting of glutathione sulfotransferase (GST) of about 26 kDa fused to the N-terminal of the cloned ORF5 encoded protein. The pET21a-5 plasmid produced a fusion protein consisting of ORF5 encoded protein fused by it's C terminal to a short polyhistidine tag providing a zinc finger motif. GST and zinc finger motif are designated for purification of the fusion proteins by the affinity chromatography. Fusion recombinant proteins were produced according to the plasmid suppliers instruction manual. Plasmids pGEX-5 and pET21a-5 were introduced into BL21(DE3) (Novagen) competent *Escherichia coli* cells, according to standard methods (Sambrook *et al.*, 1989). The transformed bacteria were grown in 2YT medium containing 2% D-glucose and 100 μg/mL of ampicillin to produce GST-ORF5 fusion protein by pGEX-5 or in M9 medium to produce ORF5-polyhistidine (ORF5-pH) fusion protein by pET21a-5. Transformed bacteria were incubated to reach logarithmic growth phase and were induced at an optical density of 1.2-1.5 at 600 nm by adding 0.5mM IPTG to the culture media. After 6 h incubation at 37°C, the cells were pelleted and resuspended in 1:25 of the initial culture volume in ice cold PBS containing 1mg/mL lysozyme and 1mM phenyl methyl sulfonyl fluoride (PMSF). After 20 min incubation at 0°C, triton X100 was added to the suspension at a final concentration of 1 per cent. Cells were disrupted by sonication after 10 min incubation on ice and the fusion protein entirely in form of inclusion bodies was washed twice with 0.5M Glycine-NaOH buffer pH 9.2. The final pellet was solubilized by denaturation in the aforementioned buffer containing 8M urea. The solubilized GST-ORF5 fusion protein was refolded by chromatography on G25 sepharose (Bio-Rad) column, then purified by affinity chromatography on glutathione-Sepharose 4B (Pharmacia) and eluted from the gel with reduced glutathione (20mM-glutathione, 150mM Tris-HCl, pH 9.6). The ORF5-pH fusion protein was purified by electroelution following separation by SDS-PAGE and then dialyzed during 24h at 4°C against 0.5 M glycine-NaOH buffer. The

protein concentration in purified antigen preparations was determined by spectrophotometry.

The recombinant fusion proteins were then analysed by SDS-PAGE and their serological identification was confirmed by Western immunoblotting, using porcine anti-PRRSV (IAF-Klop strain) serum from experimentally-infected pigs (Loemba *et al.*, 1996) and porcine anti-GST-ORF5 monospecific hyper-immune serum (Mardassi *et al.*, 1996). The Western immunoblot assay was done essentially as described previously (Mardassi *et al.*, 1994b). Both antisera strongly reacted with the recombinant fusion proteins (Fig. 2.1A lanes 5 & 6; Fig. 2.1B lanes 7 & 8). No reactivity was observed with the recombinant fusion proteins by SPF pig sera (Data not shown). The molecular masses of the recombinant fusion proteins were approximately 45 kDa for GST-ORF5, and 23 kDa for ORF5-pH, in agreement with the M_{rs} determined previously from the amino acid sequences of the IAF-Klop strain ORF5 product (Mardassi *et al.*, 1995).

1.3.3 Immunization and development of hybridomas producing MAbs for PRRSV

MAbs were prepared as described previously (Dea & Tijssen, 1989), except that P3X63 Ag8-653 myeloma cells were used and feeder cells were suppressed. We immunized mice with GST-ORF5 fusion protein, whereas ORF5-pH fusion protein was used to assess the humoral immune response of the above mice and screening the antibody secreting hybridoma by ELISA and Western immunoblotting. This strategy was chosen to avoid confusion resulting from reactivity of mice sera or hybridoma secretions with GST. The latter is a highly immunogenic protein which, in this study, could not be appropriately cleaved from the GST-ORF5 fusion protein by digestion with thrombine.

Six to eight-week-old BALB/c mice were immunized intraperitoneally with 50-60μg of GST-ORF5 fusion protein suspended in Freund's complete (first injection) or incomplete adjuvant. After three injections given at 2-week intervals, titres of sera from mice providing the immune splenocytes ranged between 256 to 1024 by indirect immunofluorescence (IIF) test using acetone fixed PRRSV-infected MARC-145 cells (Yoon *et al.*, 1995) and a commercial fluorescein-conjugated anti-mouse IgG (Boehringer Mannheim). Two weeks after the last inoculation, mice that also tested positive by Western immunoblotting against the density gradient purified PRRSV (Fig. 2.2, lane 7) received a final intravenous injection of 25μg of the recombinant antigen in PBS.

1.3.4 Screening of antibody secreting hybridomas

Screening hybridoma culture media and mouse ascitic fluids for the presence of anti-ORF5 protein antibodies by ELISA was essentially performed as previously described (Dea & Tijssen, 1989) with minor modifications. Sucrose gradient-purified virus (0.5μg of protein/well) (Mardassi *et al.*, 1994b) or gel-purified ORF5-pH fusion protein (0.1 μg of protein/well) in 0.05M-sodium carbonate buffer pH 9.6, was used to coat flat-bottomed microtiter plates. A commercial peroxidase-labelled goat anti-mouse Ig conjugate (Boehringer-Mannheim) was used to detect captured antibodies. The substrate solution consisted of 0.1% urea peroxide and 0.02% 3,3',5,5'-tetramethyl benzidine, in 10 mM citrate buffer, pH 5.0, mixed in equal volumes. After an incubation period of 10-15 min at room temperature, the reaction was stopped by addition of 50 μL 2N sulfuric acid and the absorbance values were determined at 450 nm.

Supernatants from hybridoma culture media were systematically tested by ELISA against the GST-ORF5 and ORF5-pH fusion proteins, and by IIF against PRRSV-infected MARC 145 cells. After screening of 124 antibody secreting hybridoma originating from 3 different fusions and subcloning by limiting dilution method, only five subclones were found appropriate for ascitic fluids production. These subclones

were grown to high-density, and the subisotypes of MAbs secreted in the culture medium were determined to be IgG1 by using a commercial enzyme immunoassay kit (Boehringer-Mannheim). Ascitic fluids were produced by intraperitoneal injection of 5 X 10^5 to 5 X 10^6 hybridoma cells into BALB/c mice that had been primed with Pristane (2,6,10,14-tetramethylpentadecane) (Sigma).

1.3.5 Western immunoblotting and radio-immunoprecipitation assay

The viral protein specificity of the anti-PRRSV MAbs produced in ascitic fluids was determined by Western immunoblotting and radioimmunoprecipitation (RIPA), as described previously (Mardassi *et al.*, 1994b) with minor modifications. Sucrose gradient-purified extracellular virus, as well as purified GST-ORF5 and ORF5-pH recombinant fusion proteins were resuspended in electrophoresis sample buffer in the presence of 5% β-mercaptoethanol, boiled for 4 minutes, and subjected to SDS-PAGE using a 12% polyacrylamide resolving gel. Proteins were electrophoretically transferred onto nitrocellulose membranes (pore size, 0.45μm; Schleicher & Schuell). For each strip, 5-6μg of viral or recombinant protein was used. After saturation with 5% BLOTTO (skim milk powder in 0.05 M tris-buffered saline solution), individual strips were incubated overnight at 4° C in the presence of either a 1:50 dilution of homologous anti-PRRSV porcine serum or a 1:50 dilution of mouse ascitic fluid.

Immunoprecipitation assays were conducted with concentrated preparations of radiolabelled virus. Briefly, confluent monolayers of MARC 145 cells were infected with IAF-Klop strain at a multiplicity of infection of 0.1 $TCID_{50}$ of virus per cell. At 24h postinfection, the cells were methionine-starved for 3h, then reincubated during 48 h, or until 50% of the cells showed cytopathic changes, in culture medium containing 50μCi/mL of [^{35}S]-methionine (specific activity 1,200 Ci/mmol.; Amersham). The infected cells were harvested by two freeze-thaw cycles, and radio

labelled extracellular virions were pelleted by ultracentrifugation as previously described (Mardassi *et al.*, 1994b). For immunoprecipitation, viral pellets were disrupted in the lysis buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA (ethanoldiamine tetra-acetic acid), 0.5% NP-40, 0.5% sodium desoxycholate , 0.1% SDS, 1mM PMSF, with or without 0.5mM dithiotreitrol) and aliquots (50 µL) were incubated overnight with 10µL of ascitic fluid or rabbit monospecific antisera to the ORF5 (E), ORF6 (M) and ORF7 (N) products of PRRSV, available from previous experiments (Mardassi *et al.*, 1996). The immune complexes were then adsorbed to protein A-Sepharose CL-4B beads (Pharmacia), pelleted and washed 4 times with 20mM Tris-HCl, pH 7.6, containing 150mM NaCl, 5mM EDTA and 0.1% NP-40, and twice with 20mM Tris-HCl, pH 7.6, containing 0.1% NP-40 only. The immunoprecipitated proteins were dissolved in 50 µL of electrophoresis sample buffer in the presence of 2-mercaptoethanol, analysed by SDS-PAGE and visualized by autoradiography.

1.3.6 Virus neutralization test

To determine if the anti-ORF5 protein MAbs could neutralize virus infectivity, ascitic fluids were heat-inactivated (56°C for 60 min) and serial dilutions were incubated with 100 $TCID_{50}$ of PRRSV in a microneutralization assay (Dea & Tijssen, 1989). Cytopathic changes (c.p.e.) or infected cells expressing the N protein were visualized by microscopic observation following staining by indirect immunoperoxidase (Wensvoort *et al.*, 1991b) using anti-N MAb IAF-K8 and a commercial peroxidase-conjugated anti-mouse IgG (Boehringer-Mannheim). The VN tests were done on both MARC-145 cells and primary culture of porcine alveolar macrophages (PAM cells) (Mardassi *et al.*, 1994b). The VN titres were expressed as the reciprocal of the highest serum or ascitic fluid dilution leading to total inhibition of c.p.e. and expression of viral N protein in the PRRSV- infected cultures.

1.4 Results

1.4.1 Reactivity of MAbs with PRRSV and recombinant ORF5-pH

Titres of five MAbs secreted in the ascitic fluids ranged between 1,600 to 12,800 by IIF with PRRSV-infected (IAF-Klop strain) MARC-145 cells, while no reactivity was observed to non-infected cells. By ELISA, titres of ascitic fluids ranged between 1,250 to 6,250 when tested against the recombinant ORF5-pH fusion protein (Table 2.1). In IIF tests, these MAbs reacted with variable titres to the attenuated vaccine strain ATCC VR-2332 (Collins *et al.*, 1992b) but failed to react to the European prototype LV strain of PRRSV (Table 2.2).

1.4.2 MAbs reacted specifically with the recombinant fusion ORF5 proteins and the native GP_5

All five MAbs described above reacted with a 25 kDa band of viral protein (Fig. 2.2a, lanes 2 to 5 and Fig. 2.2b, lanes 2 and 3) which was also revealed by the homologous murine and porcine anti-GST-ORF5 hyper- immune serum (Fig.2.2a, lanes 6 and 7) and the porcine PRRS convalescent sera (Fig. 2.2.b lanes 1 and 6). Similarly to the porcine anti-GST-ORF5 and porcine PRRS convalescent serum (Fig 2.1a, lanes 5 and 6), the antibodies contained in the ascitic fluids also reacted positively with the above mentioned recombinant ORF5 peptides (Fig. 2.1a, lanes 2 to 4; and Fig. 2.1b, lanes 2 to 6). For comparison, no reactivity to the 25 kDa virus protein was revealed following incubation with two previously characterized anti-PRRSV MAbs, MAbs IAF-K8 and IAF-K6 (Fig. 2.2b, lanes 4 and 5), directed against the N (15 kDa) and M (19 kDa) proteins of the IAF-Klop strain of PRRSV (Dea *et al.*, 1996). As expected, the latters also failed to react to purified GST-ORF5 and ORF5-pH recombinant fusion proteins (data not shown).

Under the electrophoresis conditions described above, all five MAbs obtained following immunization with GST-ORF5 recombinant fusion protein precipitated a 25 kDa protein corresponding to the glycosylated product of ORF5 (Fig.2.3, lanes 7 to 10), whereas monospecific rabbit antisera directed against the ORF5, 6, and 7 products precipitated proteins of 25, 18, and 15 kDa (Fig. 2.3, lanes 1 to 3), corresponding to the GP_5, M, and N proteins of PRRSV, respectively. As in the case of the monospecific rabbit antiserum directed against GP_5 protein (Fig.2.3, lane 3), the six anti-ORF5 protein MAbs coprecipitated both the GP_5 and M proteins. Similarly, rabbit anti-M monospecific antiserum precipitated both M and GP_5 proteins. This coprecipitation is explained by the association of the GP_5 with M protein into disulfide-linked heterodimers in Arteriviruses (de Vries *et al.*, 1995, Mardassi *et al.*, 1996). The N protein was also precipitated along with M or GP_5 immune complexes, but the amount was substantially smaller than the amount precipitated by the monospecific anti-N rabbit serum (Fig. 2.3, lane 1). However, neither M nor GP_5 proteins were precipitated by the anti-N hyper- immune serum. This phenomenon has already been reported and is believed to be due to a non-specific precipitation of N protein by protein A-Sepharose (de Vries *et al.*, 1995) or an unidentified interaction between the GP_5 and the N protein of PRRSV (Mardassi *et al.*, 1996). As it was demonstrated for EAV structural proteins, incorporation of 0.5mM DTT (dithiotreitol) in the RIPA lysis buffer resulted in the vanishing of non-specific coprecipitation of N protein and substantially decreased coprecipitation of M protein by the anti-GP_5 MAbs (Fig 2.3, lanes 12 to 15). Serum samples collected from SPF pigs prior to experimental infection (negative control) failed to precipitate radiolabelled PRRSV structural proteins (Fig. 2.3, lane 4).

1.4.3 MAbs specific to the ORF5 product of PRRSV neutralize viral infectivity

The five anti-GP_5 MAbs obtained in the present study were found to be neutralizing, with VN titres ranging from 32 to 128 when tested against the

homologous virus propagated in MARC-145 cells. These MAbs also neutralized, with approximately similar titres, the homologous virus propagated in PAM cells (Table 2.1). Furthermore, two of these 5 anti-ORF5 protein MAbs (MAbs IAF-8A8 and IAF-1B8) displayed neutralizing activity towards the heterologous modified-live attenuated vaccine strain ATCC VR-2332, but none of these MAbs neutralized the European prototype LV strain when tested on MARC-145 cells (Table 2.2).

1.5 Discussion

In this report we described for the first time the production and characterization of MAbs to the GP_5 of PRRSV which specifically react with the virus encoded GP_5 or the recombinant ORF fusion protein in Western immunoblotting, IIF, ELISA and RIPA. Interestingly, the five MAbs described in this report showed neutralizing activity against the homologous Québec reference IAF-Klop strain. This implies that the GP_5 protein of PRRSV has a role in virus infectivity and may function in attachment for cell receptors and/or in virus penetration into the cytoplasm of target cells. This indicates that at least one neutralizing antigenic determinant is associated with the GP_5 of PRRSV. Since all MAbs reacted with both the glycosylated and unglycosylated forms of the viral envelope protein, it appears that glycosylation is not associated with the neutralizing epitope(s) recognized by these MAbs. This phenomenon can furthermore indicate that an important linear neutralizing epitope exists in the GP_5 of PRRSV, although conformational epitopes in the protein cannot be excluded. Indeed, fusion proteins expressed in procaryotic vectors may not effectively mimic the native viral proteins due to the differences in polypeptide folding, disulfide bond formation or post-translational modifications notably N-glycosylation.

Antibody dependent enhancement (ADE) is defined by increased virus infection in presence of specific antibodies and it has been described both *in vitro* and *in vivo*

for several viruses including PRRSV (Yoon *et al,* 1996). This is attributed to the opsonization of the antigen after immune complex formation, via specific interaction between macrophages and the Fc fragment of the immunoglobulins. Interaction between the Fc fragment of murine Igs and the Fc receptor of the PAMs has not been reported so far. On the contrary, we have not observed any detectable interaction between murine Igs and uninfected PAMs in flow cytometry (unpublished data). Yoon *et al* (1996), have reported significant increases in PRRSV yield and infectivity on PAMs when virus progenies were incubated with subneutralizing dilutions of porcine specific anti-PRRSV sera but no significant ADE was observed in presence of neutralizing levels of specific anti-PRRSV porcine sera. The above observations are in agreement with our results indicating that the ADE did not interfere with VN in presence of neutralizing dilutions of anti-GP_5 MAbs. It was not in the scope of our study to establish if subneutralizing amounts of the MAbs could result in increased virus yields.

In general, the results of the present study suggest that antigenically the GP_5 of PRRSV is the counterpart of the major envelope glycoprotein of other arteriviruses (Plagemann & Moennig, 1992), notably EAV, the prototype of the genus (Chirnside *et al.,* 1995a). The ectodomain of the G_L glycoprotein of EAV which is located at its N-terminal half, contains a highly immunogenic region consisting of not more than 44 amino acids residues. An immunodominant epitope maps to this region which induces neutralizing antibody in horses. Immunizing horses with an *E. coli*-expressed linear protein encoded by this region of G_L or with a synthetic peptide representing close to 50% of this immunogenic region induced EAV-neutralizing antibody in vaccinated horses (Chirnside *et al.*, 1995a). Moreover, characterization of different neutralization-resistant escape mutant viruses with a panel of six anti-EAV neutralizing MAbs, as well as competitive binding assays, indicated that this linear immunodominant region of G_L comprises at least three interactive neutralizing epitopes (Balasuriya *et al.*, 1995a; Deregt *et al.*, 1994). Generation of such mutant viruses for PRRSV, as well as competition binding assays should permit to define

how many neutralizing epitopes are clustered on the N-terminal half of the GP_5.

The GP_5 of PRRSV is rather abundantly present in the virion and is partially exposed in association with the lipidic envelope (Mardassi *et al.*, 1996; Meulenberg *et al.*, 1995). Although it has been recently demonstrated that ORFs 2 , 3 and 4 also coded for envelope-associated glycoproteins of LV (Meulenberg *et al.*, 1996) , and that MAbs generated to the GP_4 can also neutralize viral infectivity *in vitro* (van Nieuwstadt *et al.*, 1996), these proteins are generally only weakly recognized by convalescent pig sera (Loemba *et al.*, 1996; Nelson *et al.*, 1994; Yoon *et al.*, 1995), and thus can only be considered as minor viral structural proteins whose role in the protection or recognition by immune cells is still to be determined. Several authors have demonstrated by Western immunoblotting and RIPA, using purified or concentrated virus preparations, the GP5 is the major viral envelope glycoprotein, being mostly recognized by all convalescent pig sera (Mardassi *et al.*, 1994b; Nelson *et al.*, 1993; Meulenberg *et al.*, 1995; Yoon *et al.*, 1995). We have also recently demonstrated that pigs naturally- or experimentally-exposed to PRRSV developed antibodies directed against the GP_5 as soon as 7 to 11 days postinoculation, although neutralizing antibodies are usually not detected until 3 to 4 weeks (Loemba *et al.*, 1996). We can therefore suggest that the GP_5 has the highest immunogenicity amongst the membrane-associated glycoproteins of PRRSV.

Further studies are needed in order to establish if distinct neutralizing antigenic domains are present on the GP_5 of PRRSV. Nevertheless, the results obtained from the study of the comparative reactivity of the five anti-ORF5 protein MAbs to the modified-live attenuated vaccine strain ATCC VR-2332 and the European prototype LV strain provide preliminary evidence on the existence of at least two neutralizing epitopes on the GP_5. This assumption is based on the fact that only two of the 5 MAbs tested neutralized the US strain with similar titres as the homologous strain, while all five failed to react to the LV strain. These results could be anticipated since previous genomic studies established that the amino acid sequence identity between

the Québec IAF-Klop strain and the reference US strain was 89% for the ORF5 encoded glycoprotein, whereas the predicted product of the Québec and LV strains display only 52% amino acid identities (Mardassi *et al.*, 1995; Meng *et al.*, 1995a). Of a particular interest is the recent identification within the ORF5 protein of North American field isolates of an hyper variable region with antigenic potential on the N-terminal half of the protein (Meng *et al.*, 1995a). Similar findings were also described in the cases of Canadian field isolates (Conference Research Workers in Animal Diseases, Chicago, Nov. 1996). Since three of the anti-ORF5 protein MAbs tested failed to neutralize the ATCC VR-2332 strain, we cannot exclude the possibility that they may be directed to this region. Competitive binding assays and the study of the reactivity of the MAbs with the truncated ORF5 encoded protein are in progress in order to establish the number of the immunodominant epitopes associated with the GP_5 of PRRSV.

Table 2.1. Characterization and reactivity of MAbs directed against the IAF-Klop strain of PRRS virus

MAb*	Titers of ascites determined by:				Protein specificity as defined by:	
	IIF†	ELISA‡	VN§		WB	RIPA
	MARC 145 cells	ORF5-pH fusion protein	MARC 145 cells	PAMs		
IAF-8A8	6,400	1,250	64	128	GP_5	GP_5
IAF-1B8	12,800	2,500	128	128	GP_5	GP_5
IAF-2A5	3,200	1,250	128	128	GP_5	GP_5
IAF-3A12	1,600	6,250	64	64	GP_5	GP_5
IAF-1C10	1,600	1,250	32	64	GP_5	GP_5
Murine αGST-ORF5 serum	256–1,024	1,250	64	128	GP_5	GP_5
SDOW17	100,000	-	< 8	< 8	N	N
IAF-K8	100,000	-	< 8	< 8	N	N
IAF-K3	1,600	-	< 8	<8	M	M
IAF-K6	1,600	-	< 8	< 8	M	M

* Ascitic fluids.

† Titers of PRRSV MAbs by indirect immunofluorescent test, expressed as the reciprocal of the highest dilution of ascitic fluid at which specific cytoplasmic fluorescence was observed.

‡ ELISA: endpoint dilution was defined as the reciprocal of the highest dilution of ascitic fluids producing A_{450} value > 2.5 × A_{450} value of an irrelevant murine ascitic fluid.

§ Virus neutralization: reciprocal of the highest dilution of ascitic fluids neutralizing cytopathic changes and expression of viral N protein in MARC 145 cells and primary culture of porcine alveolar macrophages by 100 $TCID_{50}$ of virus.

WB = Western immunoblotting; RIPA = Radio immuno-precipitation assay; PAM = Primary culture of porcine alveolar macrophages.

Table 2.2. Reactivity of MAbs with the US attenuated vaccines strain ATCC VR-2332 and the European prototype strain LV

	Titers of ascites determined by:			
	IIF†		VN‡ on MARC 145	
MAb*	ATCC VR-2332	LV	ATCC VR-2332	LV
IAF-8A8	6,400	< 100	32	< 8
IAF-1B8	6,400	< 100	64	< 8
IAF-2A5	1,600	< 100	< 16	< 8
IAF-3A12	1,600	< 100	< 16	< 8
IAF-1C10	1,600	< 100	< 16	< 8
Murine αGST-ORF5 serum	400	< 100	32	< 8
SDOW17	102,400	51,200	< 8	< 8
IAF-K8	51,200	51,200	< 8	< 8
IAF-K3	< 25	< 25	< 8	< 8
IAF-K6	< 25	< 25	< 8	< 8

* Ascitic fluids.

† Titers of PRRSV MAbs by indirect immunofluorescent test, expressed as the reciprocal of the highest dilution of ascitic fluid at which specific cytoplasmic fluorescence was observed.

‡ Virus neutralization: reciprocal of the highest dilution of ascitic fluids neutralizing cytopathic changes and expression of viral N protein in MARC 145 cells by 100 $TCID_{50}$ of virus.

Figure 2.1:

Reactivity of anti-PRRSV MAbs to recombinant fusion proteins GST-ORF5 (*a*) and ORF5-pH (*b*) expressed in *Escherichia coli* as determined by Western immunoblotting. The recombinant fusion proteins were purified by affinity chromatography or electroelution, separated by electrophoresis through SDS-12% polyacrylamide gels and electro-transferred to nitrocellulose membranes. Individual strips were immunostained with murine ascitic fluids or porcine anti-PRRSV hyper- immune serum.

(*a*) Immunoblots obtained with GST-ORF5 recombinant fusion protein. Lane 1 corresponds to an irrelevant mouse ascitic fluid; lanes 2, 3 and 4 correspond to the reactivity of MAbs IAF-1B8, IAF-8A8, and IAF-3A12; lanes 5 and 6 are the reactivity profiles of the porcine anti-GST-ORF5 and porcine anti-PRRSV (IAF-Klop strain) hyper- immune sera.

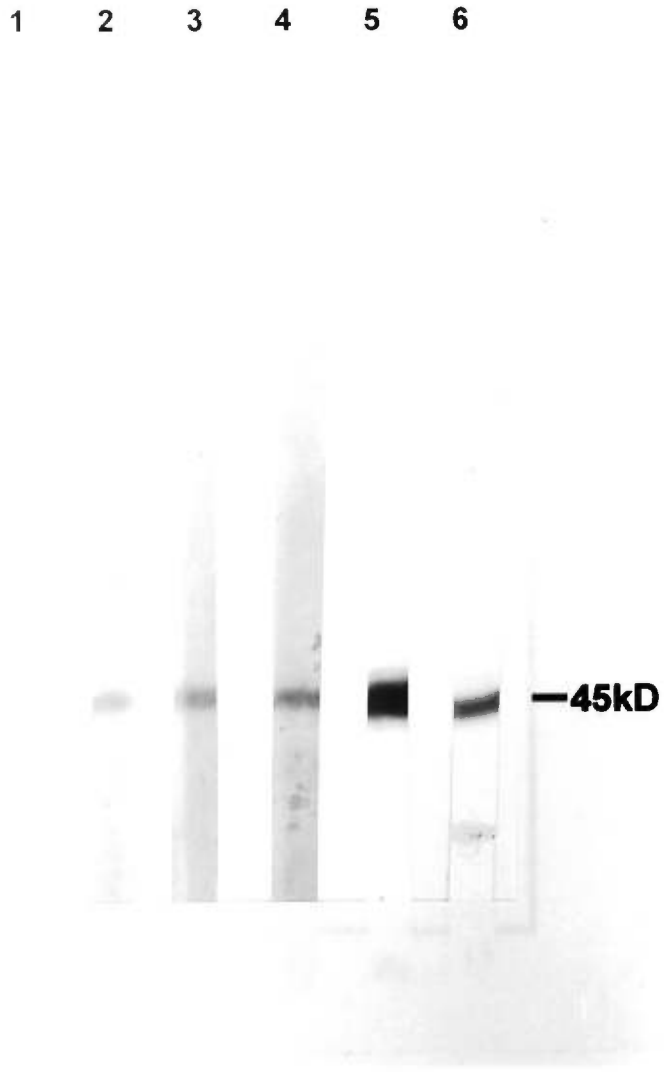
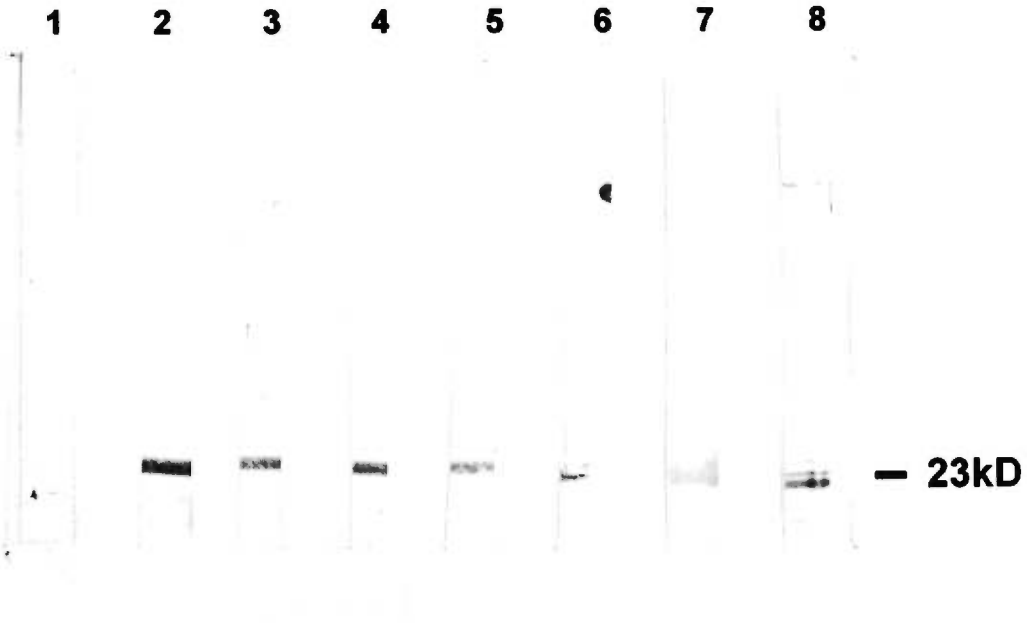

1
2
3
4
5
6
45kD

Figure 2.1

(*b*) Immunoblots obtained with ORF5-pH recombinant fusion protein. Lane 1 corresponds to the irrelevant mouse ascitic fluid; lanes 2 to 6 represent the reactivity profiles of MAbs IAF-1B8, IAF-8A8, IAF-3A12, IAF-1C10 and IAF-2A5; lane 7 and 8 are the reactivity profiles of the porcine anti-PRRSV (IAF-Klop strain) and porcine anti-GST-ORF5 hyper- immune sera.


1
2
3
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5
6
7
8
23kD

Figure 2.2:

Viral protein specificity of anti-PRRSV ORF5 protein MAbs as determined by Western immunoblotting of purified PRRSV. Sucrose gradient-purified virus (IAF-Klop strain) was solubilized in sample buffer in the presence of 2-mercaptoethanol, fractionated in 12% polyacrylamide gels, and electro-transferred to nitrocellulose. Individual nitrocellulose strips were incubated with different MAbs or immune sera

a: Lane 1: an irrelevant mouse ascitic fluid, lane 2: anti-ORF5 protein MAbs IAF-1B8; lane 3: IAF-8A8; lane4 : IAF-3A12 and lane 5: IAF-2A5 . Lanes 6 and 7 represent the reactivity profiles of murine and porcine anti-GST-ORF5 hyper-immune sera.

b: Lanes 1 and 6 represent the reactivity profiles of experimentally and naturally infected convalescent anti-PRRSV (IAF-Klop strain) porcine sera. Lanes 2 and 3 show the reactivity of MAbs IAF-1B8 and IAF-1C10. The MAbs IAF-K6 (lane 4) and IAF-K8 (lane 5) reacted to the M and N proteins, respectively.

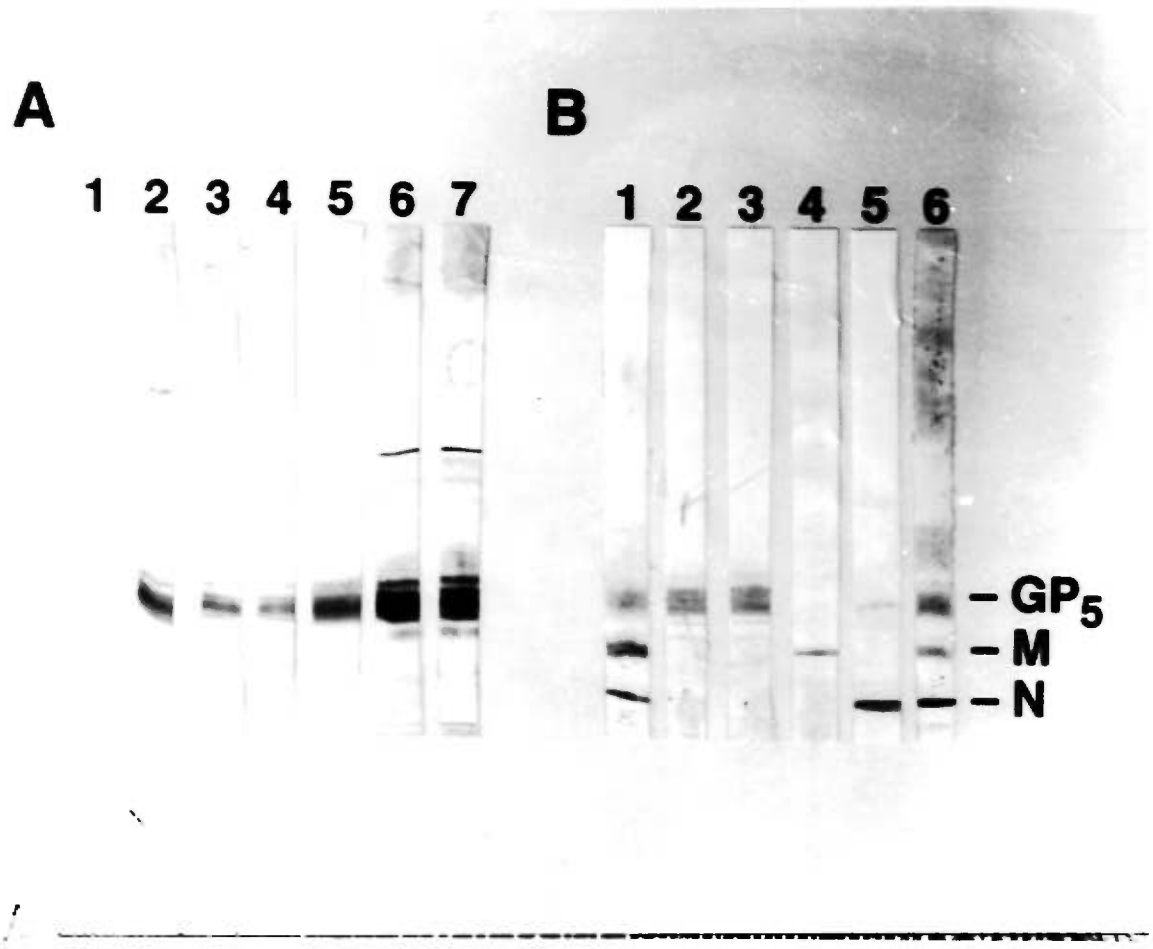
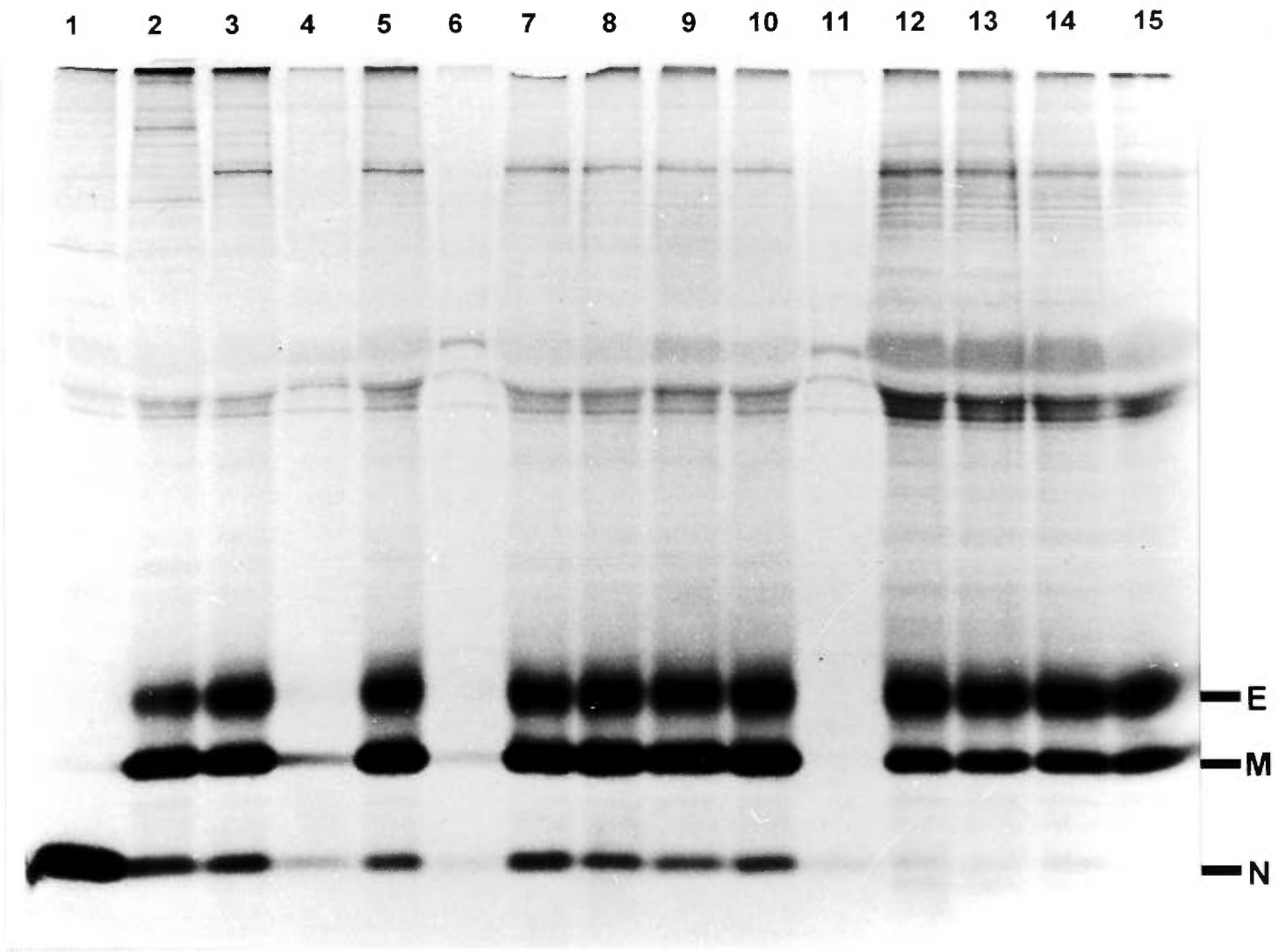

A
1 2 3 4 5 6 7
B
1 2 3 4 5 6
GP5
M
N

Figure 2.3

Immunoprecipitation of PRRSV proteins with different MAbs and monospecific rabbit and murine antisera. MARC-145 cells were infected at a multiplicity of infection of 0.1 $TCID_{50}$ per cell with the IAF-Klop strain of PRRSV and were labelled with [^{35}S] methionine for 48 hours. The supernatant of PRRSV-infected cultures were clarified and concentrated by centrifugation on sucrose gradient. The proteins were solubilized in lysis buffer in the presence (lanes 1 to 10) or absence (lanes 11 to 15) of 0.5 mM DTT. The reactivities of the various anti-ORF5 protein MAbs are indicated in lanes 7 and 12 (IAF-B8), lanes 8 and 13 (IAF-8A8), lanes 9 and 14 (IAF-3A12), and lanes 10 and 15 (IAF-1C10). Lanes 1 to 3 are the reactivity profiles of the rabbit monospecific anti-N, anti-M and anti-E (GP_5) sera, respectively. The reactivity profile of the murine anti-GST-ORF5 serum is shown in lane 5. No precipitation of the viral structural proteins was observed following incubation with the SPF porcine serum (lane 4). The location of the three major viral structural proteins are indicated on the right.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11
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E
M
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CHAPTER 2

VARIABILITY OF THE MAJOR ENVELOPE GLYCOPROTEIN (GP_5) OF PRRS VIRUS

ARTICLE 2

Genomic and antigenic variations of porcine reproductive and respiratory syndrome virus major envelope (GP_5) glycoprotein.

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2.1 Summary

The objective of the present study was to evaluate the importance of genomic and antigenic variations which may have affected the major envelope glycoprotein GP_5 of porcine reproductive and respiratory syndrome virus (PRRSV) isolates responsible for outbreaks of the disease in Québec and Ontario, in comparison with the modified-live US vaccine strain (MLV) and the European prototype LV. Nucleotide sequence analyses of the ORF5 genes showed that all of the isolates studied were heterogenous, amino acid (aa) identities varied from 88 to 99% with the MLV strain and between 51 and 54% with the LV strain. The North American strains could be placed in a subgroup distinct from that of the European prototype strain. Interestingly, amino acid substitutions of the GP_5 of tested PRRSV isolates were not uniformly scattered along the ORF5 gene but rather tended to cluster into 5 variable (tentatively named V_1 to V_5) regions. The ORF5 encoded fusion proteins were expressed in *E.coli* and used to raise strain specific hyper-immune anti-ORF5 sera in rabbits. The reactivity patterns of strain specific hyper-immune anti-ORF5 sera and a panel of four monoclonal antibodies directed against the ORF5 gene product of the Québec IAF-Klop strain of PRRSV, indicated that genomic and antigenic variations are associated with the ORF5 encoded major envelope glycoprotein. The data obtained suggest that epitopes which are not affected by the absence of carbohydrate residues are also associated with antigenic variability of the GP_5 of PRRSV.

2.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease of pigs in North America and Europe with clinical signs variable in incidence and severity. The disease affects pigs of all age groups (Dea *et al.*, 1992c; Goyal 1993; Wensvoort *et al.*, 1992a). Early signs in an affected herd consist of an influenza-like illness accompanied by hyperthermia, depression, inappetence and increased mortality in younger pigs. Severe reproductive problems can affect sows and gilts, and are characterized by increased rates of late term abortions, still-births and premature farrowing. Subclinical infections are also common and may result in diminished productivity such as decreased litter size, low conception and farrowing rates, poor appetite in sows and smaller than normal in size pigs (Goyal 1993).

The causative agent, named porcine reproductive and respiratory syndrome virus (PRRSV), is a member of a new group of small enveloped positive-stranded RNA viruses, presently classified within the genus *Arterivirus,* family *Arteriviridae*, which also includes equine arteritis virus (EAV), simian hemorrhagic fever virus, and lactate dehydrogenase elevating virus (LDV). Together with members of the family *Coronaviridae*, these viruses have been recently grouped into the order *Nidovirales* (Cavanagh, 1997). The genome of PRRSV is about 15 kb in length and contains eight open reading frames (ORFs) (Meulenberg *et al.*, 1993b). The ORF1a and ORF1b (at the 5' end) represent nearly 75% of the viral genome and code for proteins with apparent polymerase and replicase activities (Conzelmann *et al.*, 1993; Meulenberg *et al.*, 1993b). Six putative structural proteins have been identified and assigned to distinct smaller ORFs, ORFs 2 to 7, located at the 3' end of the genome (Mardassi *et al.*, 1995; Meulenberg *et al.*, 1995). The major structural proteins consist of a 25 kD envelope glycoprotein (GP_5), an 18-19 kD unglycosylated membrane protein (M), and a 15 kD nucleocapsid (N) protein, encoded by ORFs 5, 6 and 7, respectively (Mardassi *et al.*, 1995; Mardassi *et al.*, 1996; Meulenberg *et al.*, 1995). Deduced

products from the nucleotide (nt) sequences of ORFs 2, 3 and 4 genes, with respective apparent molecular masses of 30, 45, and 31 kD, have also the characteristics of membrane-associated glycoproteins (Mardassi *et al.*, 1995; Meulenberg *et al.*, 1993b and 1994). Recent findings on the characterization of structural proteins of the Lelystad virus (LV), the European prototype strain of PRRSV, indicate that expression products of the above ORFs are incorporated in virus particles and designated as GP_2,GP_3 and GP_4, respectively (Meulenberg *et al.*, 1996; Van Nieuwstadt 1996). This nomenclature was suggested during the PRRS conference in Copenhagen, August 1995 (Meulenberg *et al.*, 1997b).

While the existence of apathogenic or low-pathogenic strains of PRRSV has been demonstrated (Goyal 1993; Halbur *et al.*, 1996; Meng *et al.*, 1995a), the correlation between genomic variations and virulence variability still remains to be established for this virus. Unlike other porcine viruses, no universally recognized serotyping system is known so far. Compared to the European isolates, the North American strains of PRRSV display a high degree of variability in their ORFs 2,3,5, and 7 coding regions with less than 60% amino acid (aa) identities (Mardassi *et al.*, 1995; Meng *et al.*, 1995a and b; Murtaugh *et al.*, 1995). However, PRRSV isolates from different geographical areas of each continent are genomically closely related to each other (Gagnon & Dea 1998; Kapur *et al.*, 1996; Meng *et al.*, 1995b; Suárez *et al.*, 1996b). Among North American isolates, the ORFs 3, 4 and 5 show the highest degree of diversity as compared to other coding regions of the viral genome (Kapur *et al.*, 1996; Meng *et al.*, 1995a). Antigenic variability has also been reported by independent investigators using either field convalescent sera (Wensvoort *et al.*, 1992b) or monoclonal antibodies (MAbs) directed against the N and/or M proteins (Dea *et al.*, 1996; Drew *et al.*, 1995; Magar *et al.*, 1995, Nelson *et al.*, 1993). A recent report indicated that neutralizing MAbs to the GP_4 of LV can selectively react with the autologous strain and 1 of the 2 German isolates tested (Van Nieuwstadt *et al.*, 1996). So far, due to the lack of specific immunological probes, antigenic variability of the GP_5 of PRRSV has never been studied while such finding has been well established in

the case of the major envelope glycoprotein (G_L) of the EAV (Balasuriya *et al.*, 1995a; Glaser *et al.*, 1995).

The association of neutralizing epitopes with the G_L glycoprotein of EAV has also been demonstrated (Balasuriya *et al.*, 1993; Chirside *et al.*, 1995a; Deregt *et al.*, 1994) but until recently, the role of structural proteins of PRRSV in virus neutralization was not established. By using MAbs raised against the recombinant ORF5 protein of PRRSV expressed *E. coli*, we have recently shown that the major envelope glycoprotein GP_5 of the PRRSV is associated with virus neutralization (Pirzadeh & Dea, 1997). The antigenic variability of the GP_5 of PRRSV therefore merited investigation. In the present study we are reporting the antigenic relationship between heterogenous strains of PRRSV, as established by sequence analysis of their ORF5 genes, through investigation of their reactivity with strain-specific antisera raised against the recombinant ORF5 protein and anti-GP_5 MAbs raised to a Québec reference strain of PRRSV. The US modified live-attenuated vaccine (MLV) strain, as well as LV, were included in these comparative studies.

2.3 Materials and methods

2.3.1 Cells and virus strains.

A total of nine Canadian field isolates of PRRSV, from which seven originated from Québec and one from Ontario, were comprised in the present studies (Dea *et al.*, 1996, Gagnon & Dea 1998). The field isolates were recovered from clarified lung homogenates of dyspneic pigs and were initially propagated on porcine alveolar macrophages or MARC-145 (Kim *et al.*, 1993), a PRRSV permissive cell line (courtesy of J. Kwang, US Meat Animal Research Centre, Agriculture Research Service, USDA, Clay Centre, Nebr.). Subsequently, they were all adapted on MARC-145 cells and cloned by a limited dilution technique. The Québec reference strain IAF- Klop was plaque purified on MARC 145 cells,

according to the method already described (Mardassi *et al.*, 1995). The representative US isolate of PRRSV, MLV strain, was isolated from the commercially available modified-live vaccine (Ingelvac™, Boehringer Ingelheim Animal Health Inc. St. Joseph, Missouri, USA) on MARC-145 cells. The vaccine isolate is believed to be derived from the ATCC (American Type Culture Collection, Rockville, MD) reference strain VR-2332 (Murtaugh *et al.*, 1995). The Lelystad strain of PRRSV (LV) was kindly provided to us by G. Wensvoort (Central Veterinary Institute, Virology Department Lelystad, The Netherlands). The majority of PRRSV field isolates were passaged for not more than five times on MARC-145 cells.

The Bucyrus strain (ATCC-VR796) of equine arteritis virus (EAV), and the Purdue strain (ATCC- VR763) of porcine transmissible gastroenteritis virus (TGEV) were used as controls in the serological tests. They were grown, respectively, in rabbit kidney (RK-13) and swine testicle (ST-148) cells, as previously described (Balasuryia *et al.*, 1995a; Dea & Garzon, 1991). Convalescent porcine anti-TGEV and equine anti-EAV sera were obtained from C. Montpetit (Direction de la Santé Animale, Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, Institut Armand Frappier, Laval, Québec, Canada).

2.3.2 Reverse transcription and amplification of the ORF5 genes.

Supernatant fluids of PRRSV-infected cells were clarified and extracellular virions were concentrated by differential ultracentrifugation through a cushion of 30% sucrose (W/V), as previously described (Mardassi *et al.*, 1995). Genomic RNA of PRRSV strains were extracted from the viral pellets by one step guanidinium isothiocyanate-acid phenol method (Chomczynsky & Sacchi 1987). The ORF5 coding regions of the isolates were amplified by RT-PCR as already described (Mardassi *et al.*, 1995) using the following oligonucleotide primers:

1005PS (sense): 5'GGATCCATGTTGGGGAAATGCTTGACC3';
1005PR (antisense): 5'GGATCCGGCAAAAGTCATCTAGGG3';
ETS5L (sense): 5'GGATCCATGAGATGTTCTCACAAATTGG3';
ETR5L (antisense) : 5'GGATCCCATCTAGGCCTCCCATTG3'. These primers, containing *Bam*HI restriction site at their 5' end, corresponded to the sequence adjacent to the first ATG or the stop codon of the ORF5 coding regions of the Québec strain IAF-exp91 of PRRSV (EMBL/GeneBank accession number L40898) and of the reference European strain LV (EMBL/GenBank accession number M96262) (Mardassi *et al.*, 1995; Meulenberg *et al.*, 1993b). Oligonucleotide primers 1005PS and 1005PR were used to amplify the ORF5 coding regions of all North American isolates whereas primers ETS5L and ETR5L were used to amplify the ORF5 coding region of LV.

2.3.3 cDNA cloning and sequencing analysis

The RT-PCR amplified products were purified using the GeneClean II nucleic acid purification Kit (BIO 101, La Jolla, Ca), digested with *Bam*HI and ligated into a similarly treated pUC19 plasmid vector (Pharmacia). Alternatively, the PCR products with A overhangs were ligated into a TA cloning vector (pCRII™ vector , Invitrogen Co., San Diego, Ca), providing single 3' T overhangs at the insertion sites. The genomic region was sequenced on both strands by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using the T7 DNA polymerase (Pharmacia Biotech Inc.) in an Automated Laser Fluorescent DNA sequencer (Pharmacia LKB). To assess the error rate of the reverse transcriptase and *Taq* polymerase, clones from three different RT-PCR events were sequenced. Subsequently, the nucleotide (nt) and aa sequences were computer analyzed with the GeneWorks 2.4 program (IntelliGenetics Inc., Mountain View, Calif.). All comparisons were performed with a k-tupple length of one, and costs to open and to lengthen a gap of 2 and 4 for DNA, and of 5 and 25 for aa, respectively. The degrees of identity in nt and aa of the PRRSV isolates were deduced and compiled from sequencing data. The sequences

obtained were analysed and compared with the published sequences of the European strains LV (EMBL/GenBank accession numbers M96262) (Meulenberg *et al.*, 1993b), and those of the U.S. reference strains ATCC VR-2332 (EMBL/GenBank accession number U00153) (Murtaugh *et al.*, 1995) and ATCC VR-2385 (EMBL/GenBank accession number U03040) (Meng *et al.*, 1995b). The nt sequence accession numbers (EMBL/GenBank/DDBJ libraries) of the nine Canadian PRRSV isolates studied are as follows: U64928 for IAF-Klop; U64929 for IAF-BAJ; U64930 for IAF-DESR; U64931 for IAF 93-653; U64932 for IAF 93-2616; U64933 for IAF 94-3182; U64934 for IAF 94-287; U64936 for IAF-CM and U64935 for ONT-TS.

2.3.4 Recombinant ORF5 fusion proteins and immunization

The ORF5 coding regions of the MLV and LV strains, and those of 3 of the Canadian field isolates studied, were subcloned into procaryotic expression vector pGEX-4T1 (Pharmacia Biotech Inc.), as previously described (Mardassi *et al.*, 1996). Competent *E. coli* cells, strain BL21 (DE3) (Novagen), were transformed by the recombinant plasmid pGEX-ORF5 and grown in 2YT medium containing 2% glucose to reach a density of 1.2-1.5 at 600 nm. Transformed bacteria were induced by addition of 0.1mM IPTG to the culture medium and further incubated at 37° C for 6 hours with vigorous agitation. The GST-ORF5 fusion proteins, which accumulated within the cells in form of inclusion bodies, were purified and solubilized according to the procedure already described (Frorath *et al.*, 1992) with minor modifications. The cells were pelleted and resuspended in $^1/_{25}$ of the initial culture volume in ice cold phosphate buffered saline (PBS) containing 1mg/mL lysozyme and 1mM phenyl methyl sulfonyl fluoride (PMSF). After 20 min incubation, triton X-100 was added to a final concentration of 1 percent. Following another 10 min incubation on ice, cells were disrupted by sonication and the inclusion bodies were pelleted and washed twice with 0.5M Glycine-NaOH buffer, pH 9.2. The final pellets were solubilized in

the aforementioned buffer containing 8M urea. The solubilized recombinant GST-ORF5 fusion proteins were refolded by chromatography on G25 sepharose column (Bio-Rad) and purified by affinity chromatography on glutathion-sepharose (Pharmacia) column. Elution was performed by 20mM reduced glutathion (Sigma Chemicals) in 150mM Tris-Base, pH 9.6. Purified fusion proteins were dosed by spectrophotometric procedure against a standard pre-measured solution (Bio-Rad, protein assay Kit).

For preparation of anti-ORF5 hyperimmune sera, New Zealand albino rabbits (1600 gr; Charles River Laboratories) were intradermally inoculated with 50-60µg of recombinant GST-ORF5 fusion protein suspended in Freund's complete adjuvant (Difco) and were further boosted three times by intramuscular injection of 200µg of GST-ORF5 fusion proteins in Freund's incomplete adjuvant (Difco) at two week-intervals. Sera were collected from anesthetized rabbits by cardiac puncture and were tested for anti-PRRSV antibodies by indirect immunofluorescent test (IIF) using acetone fixed PRRSV-infected MARC-145 cells and fluorescein-conjugated goat's anti-rabbit Ig (Boehringer Mannheim, Laval, Québec, Canada), as previously described (Magar *et al.*, 1995).

2.3.5 Source of Anti-PRRSV MAbs

Four MAbs directed against the GP_5 of the Québec IAF-Klop strain of PRRSV were obtained from a previous study (Pirzadeh & Dea 1997). The polypeptide specificity of the antibodies produced in mouse ascitic fluids was confirmed by Western immunoblotting and radioimunoprecipitation tests. All four anti-GP_5 belong to the IgG1 isotype. Three of the anti-GP_5 MAbs are directed against linear neutralizing epitopes of PRRSV (IAF-8A8, IAF-1B8 and IAF-2A5) and the fourth (IAF-3B6) is apparently directed against a non-neutralizing conformational epitope. The MAb IAF-K8, also obtained from a previous study, is directed against an highly

conserved epitope of the N protein in both North American and European strains of PRRSV (Dea *et al.*, 1996). The SDOW17 MAb, also specific for the N protein of PRRSV (Nelson *et al.*, 1993) was a gift from D.A. Benfield (Department of Veterinary Science, South Dakota State University, Brookings, South Dakota). The reactivities of the field isolates were tested with the latter two MAbs in order to confirm their serological identification.

2.4 Results

2.4.1 Genomic variations between the ORF5 genes of North American and European PRRSV isolates

The aa sequences of the ORF5 product of nine Canadian field isolates of PRRSV were deduced from sequencing analyses of the RT-PCR amplified encoding genes. The sequences obtained were compared to that of the MLV strain propagated in MARC-145 cells, and the published sequences of two reference U.S. strains (ATCC VR-2332 and ATCC VR-2385) and the European reference strains LV. As shown in Fig. 3.1, the aa sequence analysis of the ORF5 genes of 12 North American field isolates (including the MLV strain), obtained from different geographical areas, revealed a total of 147 (6.1%) aa substitutions distributed along the sequence, resulting in an overall 90.9% identity among the Canadian and U.S. isolates (Table 3.1). No insertions or deletions of nt that might have resulted in aa insertion/deletion or frame shifting were found in the ORF5 of the North American isolates studied. In comparison, the levels of nt identity between the Canadian isolates and the European reference strains was only of 62-63% resulting in an overall aa identity of 51 to 54%. When the Canadian isolates were compared to each other, the aa identity varied from 88 to 99%, the ONT-TS isolate being the most variable with 88 to 93% aa identity. Among the 12 North American isolates studied, the ORF5 product of Québec field isolates

IAF-CM, IAF-DESR, IAF 94-287, IAF 93-2616 and IAF 94-3182 appeared the most conserved with less than ten aa substitutions, whereas strains MLV, ATCC VR-2332, ATCC VR-2385 and ONT-TS were the most variable with 19, 21, 17 and 15 aa substitutions, respectively (Fig. 3.1). Based on the method of Kyte and Doolittle (1982), the computer program SOAP was used to assign the sum of hydropathy values of individual residues starting at the amino terminal of the ORF5, within overlapping segments of 11 aa. In spite of several aa substitutions, the hydropathic plots of GP_5 of the North American strains remained identical with that of the Québec reference strain IAF-Klop and that of the ONT-TS strain which are shown in Fig. 3.2. Interestingly, the hydropathic profile of the GP_5 of LV shows only minor variations despite lowest level of aa identity.

The sequencing data of the PRRSV field strains studied were used to perform phylogenic analysis by Unweighted Pair Group Method with Arithmetic Mean (GeneWorks version 2.4) and accordingly, the Canadian strains where grouped in a genotype distinct from that of the reference European LV strain (Fig. 3.3). However, at least two different clades were identified among the Canadian isolates studied, the ONT-TS isolate being classified with the US MLV strain.

2.4.2 Constant and variable regions of the GP_5 protein

As expected, the aa substitutions of the GP_5 of the tested PRRSV isolates were not uniformly scattered along the ORF5 coding region and different segments showed greater variability in aa sequence. Five variable regions (tentatively named V1 to V5) were arbitrary identified (Figure 3.1), the latter being defined as genomic regions where tended to cluster aa changes for various PRRSV field isolates. Many nt changes were silent mutations (121 in total, data not shown) resulting in segments with remarkable homology which we regarded as constant (C) regions of the GP_5. The criterion for such designation was greater than 98.5% aa identity of a segment formed by 16 or more aa residues. The silent mutations generally resulted from purine substitutions (C-T; T-C), occurring mainly at

wobble base, and were randomly distributed along the coding region (68 in V regions and 53 in C regions, data not shown).

Previously investigations have suggested that the first 32 aa of the ORF5 gene product of LV strain constitute the signal sequence with a putative cleavage site situated at position 32-33 (Meulenberg *et al.*, 1995). According to the prediction method of von Heijine (1986), there is a high probability that the putative cleavage site of the Québec reference IAF-Klop strain be situated at position 25-26. With regards to the high percentage of aa identity (96%) between the N terminal of the Canadian field isolates studied, that of the MLV and the two U.S. reference strains (ATCC-VR2332 and ATCC-VR2385), the putative N terminal signal sequence of the North American strains of PRRSV seems to be located at aa 1 to 25. In this region, there was no aa identity between the North American and the European LV strain and the latters have two additional residues (threonine and glycine) inserted at positions 25 and 26, respectively (Fig. 3.1).

This region contains only 4% aa substitutions among the North American strains. The segment formed by the aa residues 26 to 41 appeared as a hypervariable region (V_1) with greater than 19% aa substitution among the North American strains which also affects the number of potential N-glycosylation sites between aa 30 to 34 (Fig. 3.4). In this region, ATCC VR-2385 and IAF 93-2616, possess no N-glycosylation site while other isolates have one to three potential N-glycosylation sites. This may in part explain the heterogeneity of the SDS-PAGE estimated apparent molecular weight of the GP_5 of the Canadian and the U.S. field isolates that has been recently reported (Magar *et al.*, 1997). The GP_5 possesses two other N-glycosylation sites at position 44 and 51 shared by all of the Canadian field isolates studied, the MLV strain, the two reference U.S. strains and the reference European strain (Fig. 3.1). The C_1 region, located between aa residues 42 to 56, is formed by 15 highly conserved aa residues showing 100% aa identity between all strains presented in this study, including LV strain. The V_2 region is constituted by 10 aa residues (position 57 to 66) and contains two clusters of aa substitutions situated at positions 57 to 59 and two aa point mutations at position

66. The C_2 region (aa 67 to 89), is formed by 23 conserved aa residues with a single aa mutation at position 73 (L changed for V) of only one Québec PRRSV isolate (IAF 94-3182). The corresponding region of LV shows 68% aa identity with the North American strains studied. The aa residues 90 to 128 constitute the V_3 region of the ORF5 gene product which contains four clusters of substitutions situated at positions 94, 101 to 102, 111 and 127 to 128. The aa mutations at positions 94 and 101 are conserved (V for I and F for Y). The C_3 region is formed by 19 highly conserved aa residues (position 129 to 147) with only one substitution associated with the MLV strain at position 137. The corresponding region of LV shows 63% aa identity with that of the North American isolates studied. The aa residues 148 to 172 constitute the V_4 region with 16 aa residues showing randomly distributed substitutions along the region. The C_4 region is a highly conserved short sequence of 16 aa (position 173 to 188) followed by the V_5 region containing 12 aa residues and randomly distributed substitutions along the region. A single aa deletion was identified at position 198 of LV strain compared to the North American strains rendering the total number of aa residues of the ORF5 encoded region of LV strain one aa greater than that of the North American isolates (201 versus 200). As can be noted in Fig.3.2, the alternating variable and constant (V and C) regions somehow correspond to the hydrophobic and hydrophilic regions of the ORF5 encoded protein.

2.4.3 Antigenic variability of the ORF5 encoded product

In order to study the antigenic variability of the GP_5 of PRRSV isolates, five of the isolates studied belonging to distinct internal branches of the deduced phylogenic tree (Fig 3.3) were selected for expression of their ORF5 product in *E. coli*. Study of the cross-reactivity of the antisera that were raised in rabbits against the recombinant GST-ORF5 fusion proteins demonstrated that each strain specific hyperimmune serum had significantly higher titres when reacted with its autologous strain in the IIF test (Table 3.2). None of these hyperimmune sera showed reactivity to EAV- or TGEV- infected cells (IIF antibody titres of < 20),

the latter being recognized by the control positive convalescent equine or porcine sera (IIF antibody titres of 320 to 640). The antiserum directed against the ORF5 protein of LV weakly recognized (IIF antibody titres of 32 to 64) the four North American strains tested, the antibody titres obtained against the homologous strain being 8 to 16 times higher. A difference of 4 to 8 dilutions was also obtained when testing each of the North American anti-ORF5 sera against LV, but the titres obtained were not significantly different from that obtained against heterologous North American isolates, except for the antiserum raised against the ORF5 product of IAF 93-653 isolate which only weakly reacted to LV, IAF-Klop and the U.S. attenuated vaccine strain. None of these five anti-ORF5 hyperimmune sera were neutralizing when tested toward the Québec reference IAF-Klop strain , the MLV and LV strains (Data not shown). Thus, in general, study of the cross-reactivity of anti-ORF5 hyperimmune sera raised against PRRSV isolates of distinct internal branches of the phylogenic tree revealed the occurrence of antigenic variability among the ORF5 product of the various PRRSV strains that may involve specific linear non-neutralizing epitopes. Apparently, these epitopes are not affected by the absence of carbohydrates residues. The data obtained also suggest that there are linear conserved epitopes among the ORF5 encoded proteins of North American and European PRRSV isolates that are recognized by polyclonal hyperimmune sera.

2.4.4 MAbs directed against the GP_5 selectively react with heterologous strains of PRRSV

To further investigate the antigenic variability of the major envelope glycoprotein GP_5 of PRRSV, the reactivities of four anti-GP_5 MAbs were tested by IIF to the nine Canadian PRRSV field isolates, the MLV and the LV strains. These MAbs were obtained from BALB/c mice that have been immunized with the *E. coli*- expressed GST-ORF5 recombinant protein of the Québec reference IAF-Klop strain. The serological identification of the various PRRSV isolates studied was confirmed by testing their reactivities to MAbs SDOW17 and IAF-K8, two anti-PRRSV MAbs directed against common antigenic determinants of the N protein of the North American and European strains (Dea *et al.*, 1996; Nelson *et*

al., 1993). As summarized in Fig. 3.5, all of the PRRSV isolates tested, including the LV strain, reacted with high titres (> 1:51200) to both anti-N MAbs. The four anti-GP_5 MAbs reacted with approximately same titres (1:1600 to 1:6400) to the MLV strain, but failed to react with MARC-145 cells that have been infected with LV. None of the six anti-PRRSV MAbs tested showed reactivity to EAV- or TGEV- infected cells (IIF antibody titres of < 20). On the other hand, the four anti-GP_5 MAbs showed various reactivity profiles to the Canadian isolates studied and accordingly, the latter could be subdivided into three subgroups. The isolates classified in subgroup 1 comprised the Québec reference strain IAF-Klop and five other Canadian field isolates (IAF 93-2616, IAF-DESR, IAF-CM, IAF 94-3182, and ONT-TS), as well as the U.S. attenuated MLV strain, which reacted positively in IIF tests with all of the four anti-GP_5 MAbs. The second antigenic subgroup comprised two other Québec isolates (IAF 93-653 and IAF-BAJ) which were selectively recognized by MAbs IAF-8A8, IAF-1B8 and IAF-3B6, but not by IAF-2A5. The remaining Québec isolate IAF 94-287 was assigned to the subgroup 3, as it reacted with the MAbs IAF-8A8 and IAF-1B8 but neither with IAF-2A5 nor with IAF-3B6. It should be noted that in some cases, the IIF titres of MAbs were lower when they were tested with heterologous strains as compared to the homologous strain (IAF-Klop). The reactivity of a MAb was considered as positive when a specific fluorescence with PRRSV-infected cells was obtained at a minimum reciprocal dilution of 1:400.

2.5 Discussion

It has been shown in this report that a relatively high degree of variability is associated with the major envelope GP_5 glycoprotein of PRRSV, and while the North American strains can be classified in a genotype distinct from that of the European strains with regards to their ORF5 coding sequence, all Canadian isolates with the exception of ONT-TS were clustered together, indicating their close genetic relatedness. These findings are in agreement with the results obtained by other investigators in relation with the U.S. and European isolates of PRRSV (Dea *et al.*, 1996; Kapur *et al.*, 1996; Meng *et al.*, 1995b; Murtaugh *et al.*,

1995) and EAV (Balasuriya *et al.*, 1995b; Glaser *et al.*, 1995; St-Laurent *et al.*, 1997). It was also demonstrated that antigenic diversity of the GP_5 glycoprotein of North American field isolates of PRRSV is not associated with an extensive degree of aa sequence heterogeneity, but the amino acid substitutions appeared to cluster into five highly variable regions of the ORF5 gene which may potentially correspond to exposed antigenic domains. It is noteworthy that no correlation could be established between the phylogenetic relationships of the various PRRSV isolates analyzed and antigenic subgroups as defined by their reactivities to hyperimmune anti-ORF5 sera and a set of four neutralizing MAbs directed against the *E. coli*-expressed ORF5 product of the Québec reference IAF-Klop strain. The data obtained further suggest that epitopes which are not affected by the absence of carbohydrate residues are also associated with antigenic variability of the GP_5 of PRRSV.

Like most RNA viruses, notably the EAV (Balasuriya *et al.*, 1995b; Glaser *et al.*, 1995; St-Laurent *et al.*, 1997), the PRRSV is also genomically heterogenous and there is ample evidence that strain variations occur in PRRSV. The aa identity of the ORF5 gene product of seven U.S. isolates of PRRSV was reported to vary from 88% to 97% (Meng *et al.*, 1995a). Similarly, the degree of identity between the ORF5 encoded G_L protein of EAV field isolates varied between 85.7% and 99.7%, a highest degree of identity was found between the North American isolates (Balasuriya *et al.*, 1995b). These results have been confirmed by a recent report indicating that the North American isolates of EAV are included in a clade apart from that of the European prototype of EAV (St-Laurent *et al.*, 1997). The antigenic variability of the G_L protein of EAV has also been established by the selective reactivity of different field isolates with anti-G_L MAbs. The genomic variability may be attributed to the lack of proof-reading function of viral RNA dependent RNA polymerase which induces errors when copying the viral genome. The genomic variations contribute to the emergence of antigenic variants of the virus which in turn results in an effective mechanism for evading host's immune surveillance. Concurrence of variable regions with the hydrophilic domains of GP_5

can be the result of host's selective humoral immune response directed against the exposed domains of this envelope glycoprotein which in turn favors antigenic drifts. As in the cases of EAV (Balasuriya *et al.,* 1993; Chirnside *et al.,* 1995a; Deregt *et al.,* 1994; Glaser *et al.,* 1995) and LDV (Coutelier & Van Snick 1988), the ORF5 encoded envelope glycoprotein of PRRSV has been found to be associated with virus neutralization (Pirzadeh & Dea 1997) and therefore, contains immunologically important domains. Clinical manifestations of PRRS are diverse and complex and they depend in part on the immune status of the host. In theory, mutation within the antigenic domains of viral proteins may contribute to the development of the chronic form of the disease and eventually a persistent infection. In the case of PRRSV, it has been previously suggested that the less virulent U.S. isolates of PRRSV had the highest genomic variability within their ORFs 2 to 4 coding regions (Meng *et al.,* 1995a). In the present study, the two U.S. reference strains analyzed showed greater variability of their ORF5 genome compared to the Canadian isolates. This is merely a reflection of their comparison with a consensus sequence perceived from a group majorly formed by Canadian strains and therefore cannot be regarded as an absolute index for their mutability.

In our experiments, the rabbit anti-ORF5 sera obtained from animals immunized with the unglycosylated linear form of the GP_5 expressed in *E. coli* reacted positively with the native form of the viral protein in IIF test. This further indicates that the unglycosylated regions of the GP_5 are also implicated in antigenic domains of the ORF5 encoded protein. It has been also demonstrated that while strain specific anti-ORF5 sera produced in rabbits cross-reacted with the heterologous strains, they gave highest titres when tested in IIF with the autologous strain of PRRSV. This can be due to the concomitant association of common and strain-specific epitopes with the GP_5 of the five strains of PRRSV used in our experiments. The shared epitopes are implicated in the cross-reactivity of the strain-specific antisera while strain-specific epitopes resulted in higher titres when these antisera were tested with the autologous strains of the virus. The titer variation of monospecific rabbit antisera can also be explained by the diminished

affinity of the antibodies directed against a single epitope due to the point-mutation of the latter in different strains. In rabbits, further experiments such as competitive ELISA with monospecific antisera are needed to elucidate this question. In mice, the reactivity patterns of a panel of four MAbs with different strains of PRRSV suggested the possibility of at least three distinct epitopes associated with the GP_5. It can be anticipated that the two MAbs IAF-8A8 and IAF-1B8 are directed against the same or two closely located neutralizing epitopes since all North American PRRSV strains tested were simultaneously recognized by both MAbs (Fig. 3.5). The MAb IAF-2A5 is apparently directed against a distinct neutralizing epitope since it failed to react or reacted only weakly with half of the strains tested, whereas the MAb IAF-3B6 recognized a non-neutralizing conformational epitope. It is noteworthy that a high degree in nt and aa identity between strains does not necessarily result in similar reactivity patterns with MAbs and, only one point mutation in the epitope recognizable by a MAb may change the reactivity of the strain with that MAb. For example, while the two Québec isolates IAF-Klop and IAF-BAJ have 99% identity in nt and aa sequences for their ORF5 gene product, they showed different reactivity patterns with the MAb IAF-2A5 and inversely, the ONT-TS and LV with only 63% identity in aa sequence of their M and N proteins shared identical reactivity patterns with four MAbs directed against N and two MAbs directed against the M proteins of PRRSV (Dea *et al.*, 1996).

Based on the aa sequences and hydropathy plots the ORF5 proteins of distinct LDV, PRRSV and EAV isolates, a model has been proposed to explain the molecular structure of the major envelope glycoprotein of the arteriviruses (Plagemann, 1996). As demonstrated in the present study, the ORF5 protein possesses a potential signal peptide and three potential internal transmembrane segments (Plageman 1996). After cleavage of the signal peptide, a segment of only about 30 aa of the LDV and PRRSV ORF5 proteins with one to three potential N-glycosylated sites would project externally (Faaberg & Plagemann, 1995; Mardassi *et al.*, 1996; Meulenberg *et al.*, 1995), which may explain the smoothness of the outer surface of the virions. At present, no data relatively to the

location of the epitopes associated with the GP_5 of PRRSV and LDV is available. However, there have been several reports on the location of the antigenic domains and neutralizing epitopes of EAV (Balasuriya *et al.*, 1995a; Chirnside *et al.*, 1995a; Glaser *et al.*, 1995). It has been clearly demonstrated that the ectodomain of the G_L glycoprotein of EAV (approximately 95 aa in length) which is located at its N-terminal half, contains a highly immunogenic region consisting of not more than 44 amino acids residues. An immunodominant epitope maps to this region which induces neutralizing antibody in horses. Immunizing horses with an *E. coli*-expressed linear protein encoded by this region of G_L or with a synthetic peptide representing close to 50% of this immunogenic region induced EAV-neutralizing antibody in vaccinated horses (Chirnside *et al.*, 1995a). Moreover, characterization of different neutralization-resistant escape mutant viruses with a panel of six anti-EAV neutralizing MAbs, as well as competitive binding assays, indicated that this linear immunodominant region of G_L encompasses three overlapping or closely adjacent neutralizing epitopes (Balasuriya *et al.*, 1995a and 1997; Deregt *et al.*, 1995). In agreement, recent amino acid analysis of the G_L protein of Canadian, American and European EAV isolates revealed 2 major variable regions encompassing residue positions 61 to 104, predicted to be located in the ectodomain of the protein (St-Laurent *et al.*, 1997). It remains to be demonstrated if V1 and V2 regions of the GP_5 of PRRSV identified in the present study are the counterparts of these highly variable regions of EAV bearing major neutralizing epitopes of the EAV (Chirnside *et al.*, 1995a, Balasuriya *et al.*, 1995a and 1997). The V1 region identified for PRRSV isolates also displayed difference in glycosylation patterns between strains but it's significance remains to be established. Since all anti-ORF5 MAbs tested were found to be directed to linear neutralizing determinants not affected by the absence of carbohydrate residues (Pirzadeh & Dea 1997), they may be directed to epitopes located in other regions of the GP5 envelope glycoprotein or in the vicinity of the V1 region. Experiments are in progress with the truncation of ORF5 encoded protein to localize the approximate location of each epitope associated with the GP_5 of PRRSV and generation of neutralization resistant escape mutants which would indicate with precision the sequence of the neutralizing epitopes associated with the ORF5 encoded glycoprotein of PRRSV.

Table 3.1. Percentage of identity in nucleotides and amino acids between the ORF5 products of different PRRSV isolates.

PRRSV Isolate	IAF-Klop	MLV	ATCC VR-2332	ATCC VR-2385	IAF-BAJ	IAF-DESR	IAF 93-	IAF 94-287	IAF 93-653	IAF 94-3182	IAF-CM	ONT-TS	LV
IAF-Klop		89%	89%	88%	99%	94%	94%	94%	94%	93%	99%	90%	63%
MLV	85%		99%	93%	89%	90%	89%	91%	90%	91%	85%	92%	63%
ATCC VR-2332	85%	99%		92%	89%	90%	89%	91%	90%	91%	85%	92%	63%
ATCC VR-2385	87%	90%	90%		88%	89%	88%	90%	89%	90%	87%	92%	63%
IAF-BAJ	99%	86%	86%	88%		94%	94%	94%	94%	94%	99%	90%	63%
IAF-DESR	92%	88%	88%	89%	92%		94%	94%	94%	95%	92%	91%	62%
IAF 93-2616	93%	89%	89%	88%	94%	93%		96%	93%	94%	89%	91%	62%
IAF 94-287	92%	90%	90%	90%	93%	94%	94%		94%	94%	92%	93%	62%
IAF 93-653	91%	87%	87%	89%	92%	93%	94%	94%		94%	91%	91%	62%
IAF 94-3182	92%	89%	89%	90%	93%	94%	93%	94%	92%		92%	91%	62%
IAF-CM	99%	89%	89%	88%	99%	94%	94%	94%	94%	93%		90%	60%
ONT-TS	88%	92%	92%	93%	88%	90%	91%	93%	90%	90%	88%		63%
LV	53%	53%	53%	54%	53%	52%	52%	53%	51%	52%	53%	54%	

Top right side: % identity in nucleotides, Bottom left side: % identity in amino acids.

Table 3.2. Reactivity of strain-specific rabbit hyperimmune sera to the recombinant fusion protein GST-ORF5 to 5 different strains of PRRSV, EAV and TGEV

Anti-ORF5 sera to	IIF antibody titers to isolate[a]						
	LV	IAF-Klop	MLV[b]	IAF 93-653	ONT-TS	EAV[c]	TGEV[d]
LV	512	64	32	64	32	< 20	< 20
IAF-Klop	128	1024	128	512	256	< 20	< 20
MLV	256	256	1024	256	256	< 20	< 20
IAF 93-653	64	64	32	512	256	< 20	< 20
ONT-TS	128	128	128	512	1024	< 20	< 20

[a] Antibody titers of rabbit hyperimmune sera expressed as the reciprocal of the highest dilution of serum at which specific cytoplasmic fluorescence was observed.
[b] MLV= U.S. attenuated vaccine strain of PRRSV;
[c]EAV = Bucyrus strain of equine arteritis virus;
[d]TGEV = Purdue strain of swine transmissible gastroenteritis virus.

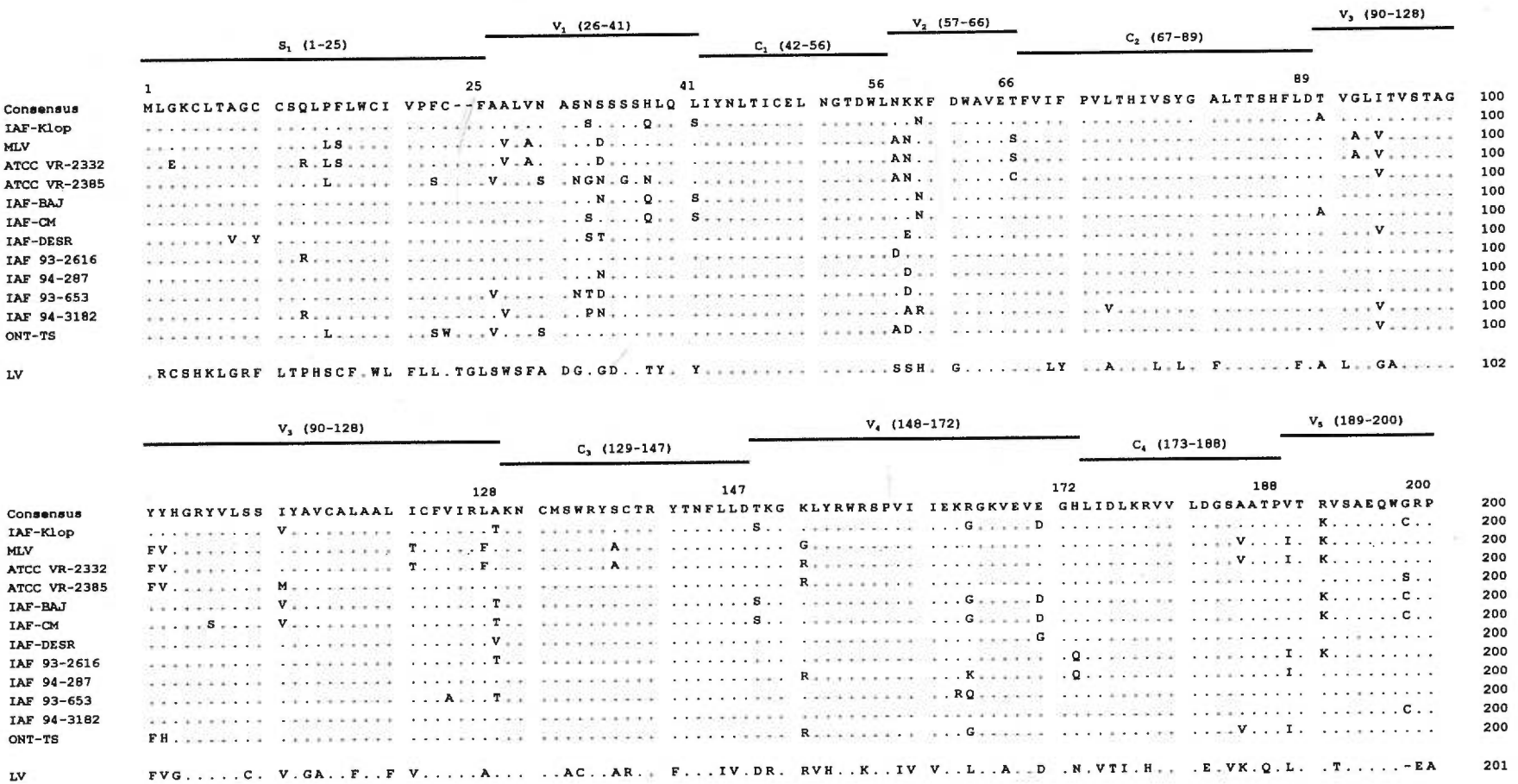

Figure 3.1

Alignment of the deduced amino acid sequences of the ORF5 encoded proteins of nine Canadian field isolates of PRRSV, and comparison to that of the U.S. attenuated MLV strain, two U.S. reference strains (ATCC VR-2332 and ATCC VR-2385), and the European reference strain LV. All sequences are compared with the consensus which is deduced from the ORF5 products of the North American strains; dots indicate the same aa as in the consensus and deletions are indicated by hyphens (-). The putative signal sequence (SS), the variable regions (V1, V2, V3, V4 and V5) and constant regions (C1, C2, C3, and C4) are indicated above the sequences.

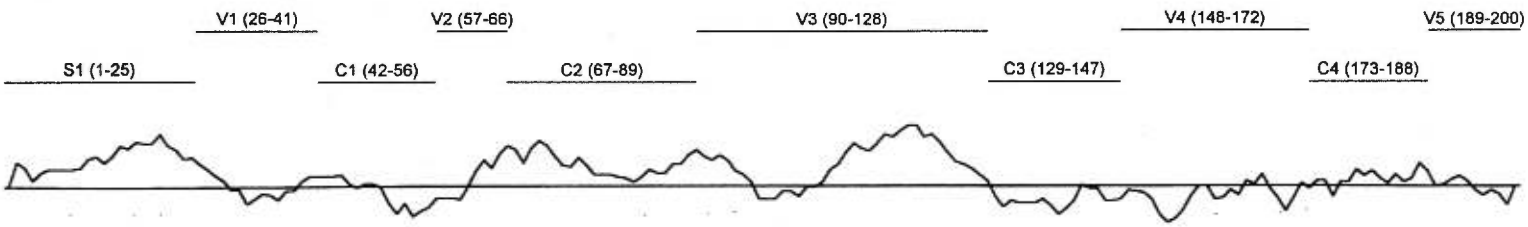

Figure 3.1 bis

Hydrophobicity profile of the ORF5 translation product of Québec's reference strain (IAF-Klop). The putative signal sequence, the variable regions (V1-V5) and constant regions (C1-C4) and the corresponding aa positions are indicated above the graph

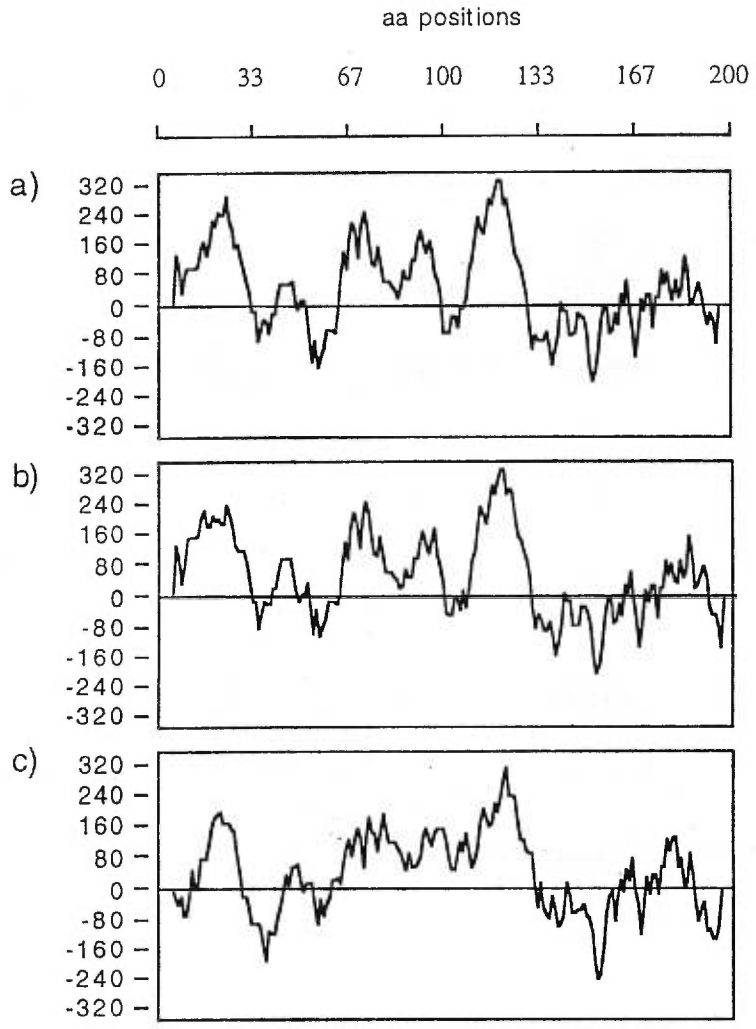

Figure 3.2
Hydropathy profiles of the ORF5 translation products of the Quebec's reference strain IAF-Klop (a), the ONT-TS strain (b), and the European prototype strain LV (c). The profiles were determined by the method of Kyte and Doolittle (1982) for the average hydropathy of overlapping segments of 11 aa shown on the vertical axis. The aa residue positions are shown above the graph's frame. Middle line separates the hydrophobic regions (above the line) from the hydrophilic regions (below the line). The alternating variable and constant regions somehow correspond to the hydrophilic and hydropathic regions of the ORF5 gene product.

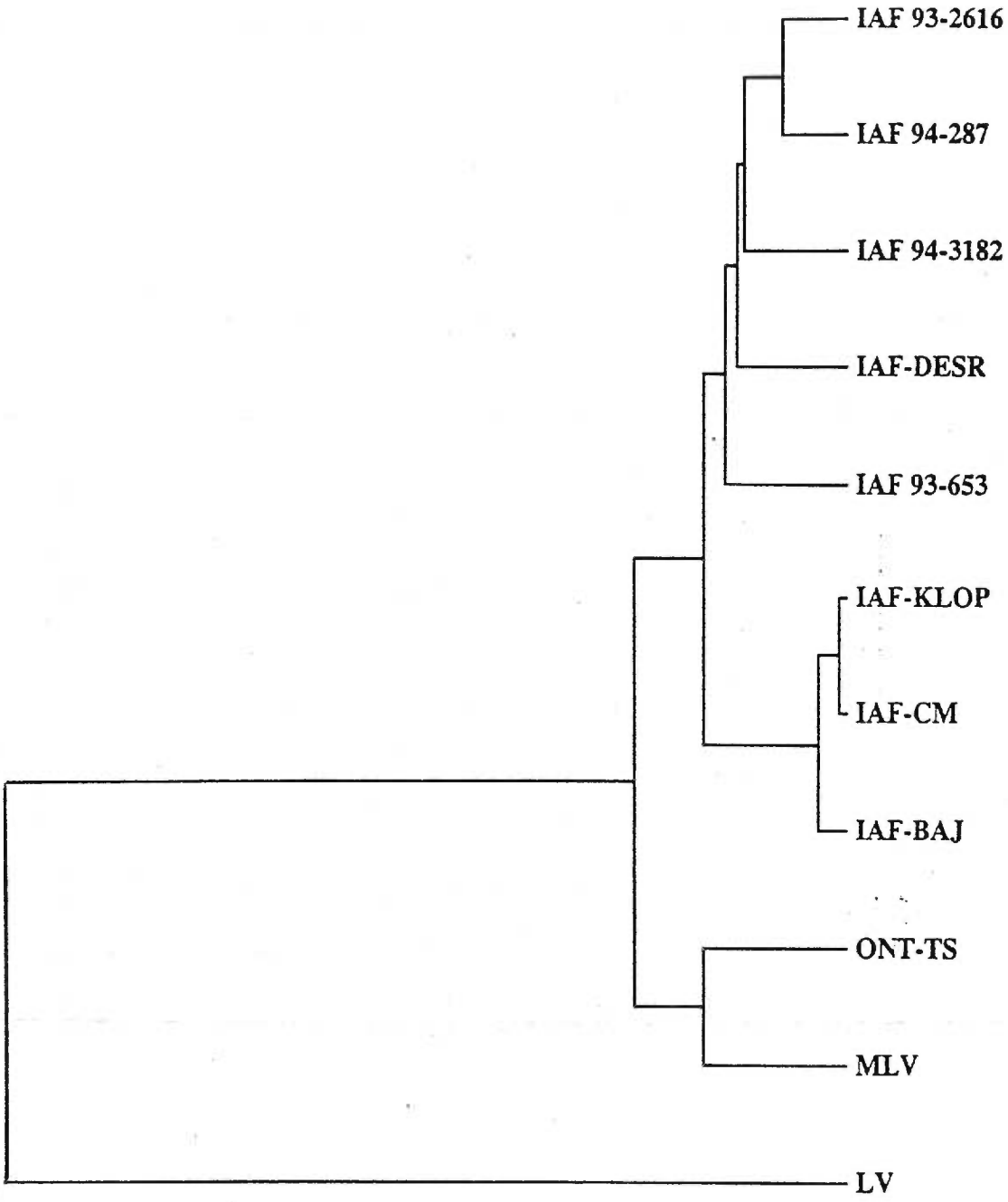

Figure 3.3

Phylogenetic relationship of nine Canadian field isolates, the MLV and the prototype European LV strains of PRRSV based on the aa sequence of their ORF5 product. The horizontal lines connecting one sequence to another are proportional to the estimated genetic distance between the sequences.

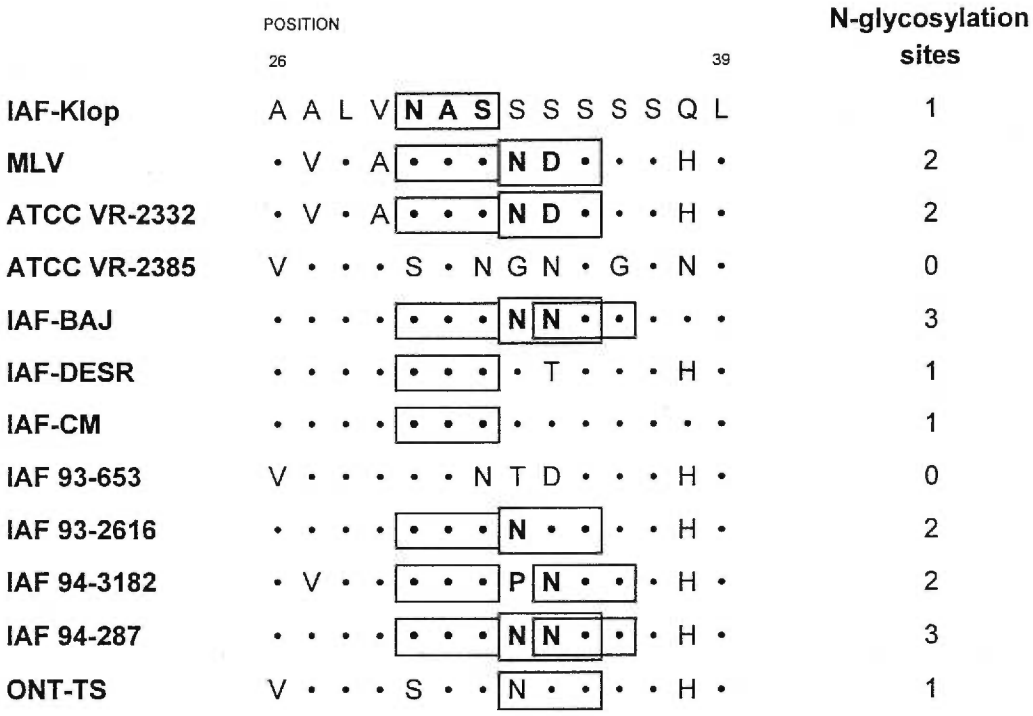

Figure 3.4
Detail presentation of the hypervariable (V1) region of the GP5 situated near the N terminal of the ORF5 (position 29 to 39), affecting the number of potential N-glycosylation sites (Boxes). Four other variable regions showing a lesser degree of variability are also associated with the coding region (data not shown).

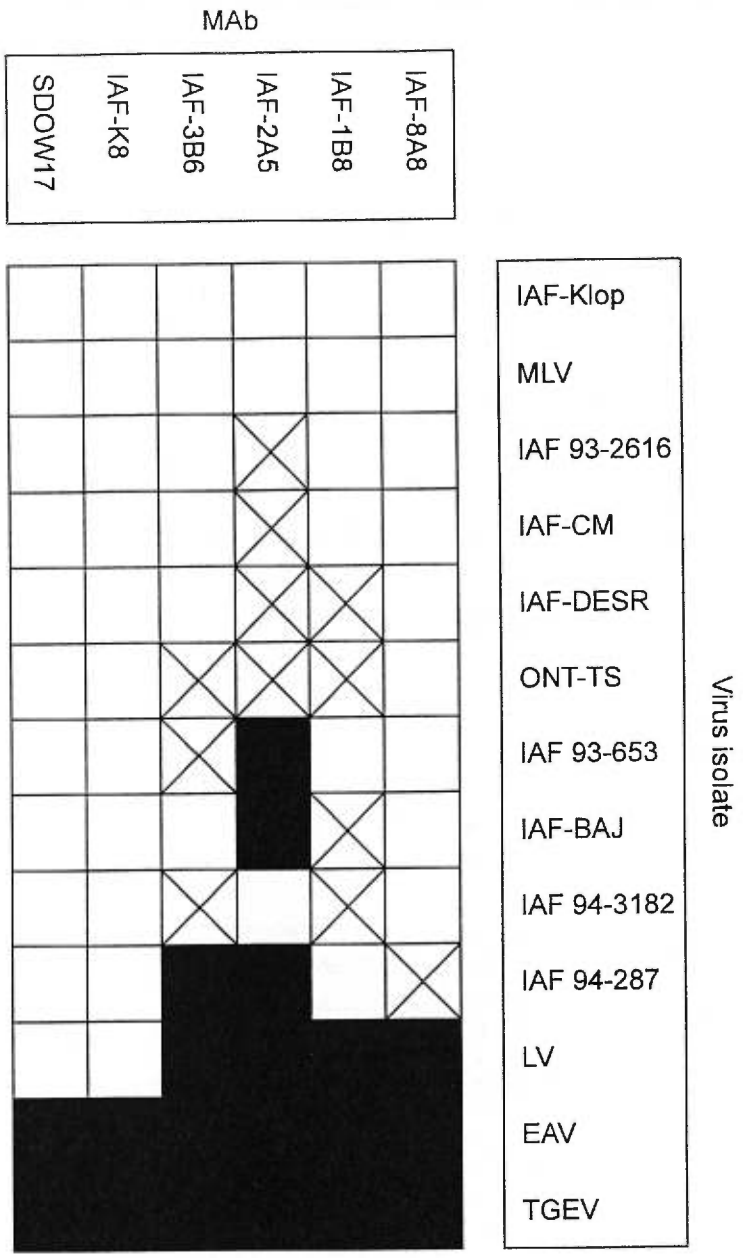

Figure 3.5
Indirect immunofluorescent titres of anti-E glycoprotein MAbs to different strains of PRRSV. Reactivity of a panel of 4 monoclonal antibodies with the GP5 glycoprotein of 11 strains of PRRSV was tested by indirect immunofluorescence. Three of these MAbs (8A8, 1B8 and 2A5) are directed against the neutralizing epitopes of PRRSV and the fourth (3B6) is directed against a non-neutralizing conformational epitope. Reactivity of the viral strains are expressed as the reciprocal of the highest dilution of ascitic fluid giving a positive cytoplasmic fluorescence with PRRSV-infected MARC-145 cells. No reactivity was observed with the Lelystad virus, Bucyrus strain of EAV and the Purdue strain of TGEV.

☐ IIF titres of 1,600 to 12,800; ☒ IIF titres of 400 and 800; ■ IIF titres of < 100.

CHAPTER 3

IMMUNOGENICITY OF THE MAJOR ENVELOPE GLYCOPROTEIN (GP_5) OF PRRS VIRUS

ARTICLE 3

Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus

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3.1 Summary

The ORF5-encoded major envelope glycoprotein (GP_5) of porcine reproductive and respiratory syndrome virus (PRRSV) is one of the three major structural proteins of the virus. While some porcine convalescent sera and monoclonal antibodies directed against GP_4 and GP_5 have the capacity to neutralize the virus *in vitro*, the protein specificity of porcine neutralizing sera has not yet been established. DNA immunization with a plasmid encoding the GP_5 of PRRSV under control of human cytomegalovirus promoter induced anti-GP_5 specific neutralizing antibodies in pigs and BALB/c mice. The GP_5 protein specificity of neutralizing sera was confirmed by immunoblotting and ELISA. Peripheral blood mononuclear cells obtained from DNA vaccinated pigs underwent blastogenic transformation in the presence of *E. coli*-expressed recombinant ORF5-encoded protein indicating the specificity of cellular immune response to GP_5. Following a massive intra tracheal challenge with the virulent IAF-Klop strain of PRRSV, DNA vaccinated pigs were protected from generalized viremia and the development of typical macroscopic lung lesions which were observed in unvaccinated, virus challenged controls, as well as in pigs that were immunized with *E.coli*-expressed GST-ORF5 recombinant fusion protein. Interstitial pneumonitis and broncho-alveolitis were remarkably milder in the case of DNA vaccinated animals. These results suggest that the GP_5 of PRRSV is a good candidate for a subunit recombinant-type vaccine.

3.2 Introduction

In spite of remarkable progress in developing vaccines that protect against viral pathogens, the conventional approaches have their own finite disadvantages. Live attenuated viral vaccines are generally quite effective as they mimic a natural infection, but apart from routine concerns such as tissue culture contaminants, they require special handling to maintain viability. A serious disadvantage of such vaccines is their pathogenicity in immunosuppressed recipients exposed to environmental stress, such as poor housing and over-crowding often prevailing in intensive animal raising operations, and can be of great concern in veterinary medicine where clinical outbreaks are sometimes reported shortly after prophylactic immunization. Administration of live modified vaccines may also result in virus persistence which in turn, contributes to generation of mutants due to the selective immune pressure on the resident variants. Persistently-infected animals may eventually shed newly generated mutants, specially in case of unstable pathogens such as RNA viruses, that may be responsible for new outbreaks. Production and purification of large quantities of viral particles for use in whole viral inactivated vaccines or their immunogenic structural proteins is economically unfeasible for low yield viruses such as porcine reproductive and respiratory syndrome virus (PRRSV). Being the apparent choice for such pathogens, the recombinant type vaccines require precise experimental data relating to the viral immunodominant and neutralizing antigenic domains.

PRRS is a spread disease found in swine farms in most parts of the world. It is characterized by reproductive failure such as late-term abortions in sows and by a respiratory illness and mortality in young pigs (Collins *et al.*, 1992a; Dea *et al.*, 1992c; Halbur *et al.*, 1996; Rossow *et al.*, 1994a). The nucleotide sequence, genomic organization and replication strategy of PRRSV are related to those of a group of small, enveloped, positive sense, single stranded RNA viruses including murine lactate dehydrogenase elevating virus (LDV), equine arteritis virus (EAV) and simian haemorrhagic fever virus which are presently classified within the

family *Arteriviridae,* order *Nidovirales* (Cavanagh, 1997). The genome of PRRSV is about 15 kb in length and contains eight open reading frames (ORFs). The ORF1a and ORF1b, situated at the 5'end of the genome, represent nearly 75% of the viral genome and code for proteins with apparent replicase and polymerase activities (Meulenberg *et al.,* 1993b). Six putative structural proteins have been identified and assigned to distinct smaller ORFs, ORFs 2 to 7, located at the 3' end of the genome (Mardassi *et al.,* 1995 & 1996; Meulenberg *et al.,* 1993b). The virion contains three major structural proteins, a 25 kDa envelope glycoprotein (GP_5), an 18-19 kDa unglycosylated membrane protein (M), and a 15 kDa nucleocapsid (N) protein, encoded by ORFs 5, 6 and 7, respectively. In addition, the translation products of the ORFs 2, 3 and 4, with respective apparent molecular masses of 30, 45, and 31 kDa, have also characteristics of membrane-associated glycoproteins (Mardassi *et al.,* 1995; Meulenberg *et al.,* 1995). Recent findings on the characterization of structural proteins of the Lelystad virus (LV), the European prototype strain of PRRSV, indicate that expression products of the above ORFs, designated as GP_2,GP_3 and GP_4, are incorporated into virus particles (Meulenberg *et al.,* 1996; Van Nieuwstadt *et al.,* 1996). While pigs develop neutralizing antibodies 4-6 weeks after exposure to virus, the protein specificity of such neutralizing antibodies is yet to be established. Monoclonal antibodies (MAb) specific to GP_4 of PRRSV neutralize the virus *in vitro* (Meulenberg *et al.,* 1997; Van Nieuwstadt *et al.,* 1996), but the reactivity of convalescent pig sera with the latter protein is not constant (Meulenberg *et al.,* 1995 & 1997a). The ORF4 gene of a North American strain of PRRSV has been also cloned and expressed in *E.coli* (Kwang *et al.,* 1994). Only 65% of PRRSV-positive sera obtained from affected pig farms reacted positively by immunoblotting with the recombinant ORF4 encoded protein. Therefore the involvement of ORF4 encoded protein in inducing neutralizing antibodies following PRRSV infection is yet to be determined.

We have recently established that MAbs derived from spleen cells of mice immunized with the recombinant ORF5-encoded protein expressed in *E. coli* neutralized PRRSV *in vitro* (Pirzadeh & Dea, 1997) and therefore, as for other

Arteriviruses, notably EAV (Balasuriya *et al.*, 1993; Chirnside *et al.*, 1995) and LDV (Coutelier *et al.*, 1988), the ORF5-encoded glycoprotein of PRRSV is associated with neutralizing epitopes. However, results of our previous studies indicated that immunizing with the same antigenic preparation did not induce neutralizing antibodies in pigs (Loemba *et al.*, 1996). The purpose of the present work was to study the immunogenecity of the GP_5 of PRRSV in pigs and to establish if animals exposed to the native form of the protein by mean of DNA immunization develop specific neutralizing and protecting antibodies.

3.3 Materials and Methods

3.3.1 Experimental animals

Twelve crossbred F1 (Landrace X Yorkshire) SPF piglets weaned at three weeks of age were obtained from a breeding farm in the province of Québec. The breeding stock and piglets were tested and proven to be seronegative for PRRSV, encephalomyocarditis virus (EMCV), porcine parvovirus (PPV), haemagglutinating encephalomyelitis virus (HEV), transmissible gastroenteritis virus (TGEV) and *Mycoplasma hyopneumoniae*. The piglets used in this study were from two different litters and randomly divided into four experimental groups (G1-G4). Each group of three piglets were allocated to separate isolation rooms in facilities equipped with microorganism-free filtered in-flowing and out-flowing air system. The animals were cared for and housed according to the specification of the level B confinement of the Canadian Council on Animal Care. Piglets were given 100 mg iron dextran at birth and received a dose of 60 mg/kg of oxytetracycline (Rogar) by IM injection at day 20 immediately prior to transfer to isolation units and left during seven days for acclimatization. They were fed commercial food and water *ad libitum*.

Six week-old female BALB/c and CD-1 mice were purchased from Charles River Laboratories and separated in groups of five mice per cage which were equipped with individual filtered air channels. There were three groups of mice per

each linage (G1-G3). There were two G3 groups, one for CD-1 and one for BALB/c, therefore there were 30 mice in total, 15 CD1 and 15 BALB/c.

3.3.2 Virus and challenge

The Québec cytopathic strain IAF-Klop (Mardassi *et al.,* 1995) used in this study was initially isolated from an acute case of PRRS and propagated in MARC-145 cells, a clone of MA-104 cells highly permissive to PRRSV (Kim *et al.,* 1993), graciously provided to us by J. Kwang (US Meat Animal Research Centre, USDA, Agricultural Research Service, Clay Centre, Nebraska). Virus titration was done by end-point dilution using immunoperoxidase on monolayer assay (IPMA) (Wensvoort *et al.,* 1991b) and virus titres were expressed in tissue culture infective dose 50 ($TCID_{50}$) per mL, as previously described (Dea & Tijssen, 1989). In order to verify the virulence of the virus strain and eliminate MARC-145 cellular proteins may have been copurified with the virus, PRRSV strain IAF-Klop was subjected to one passage on primary cultures of porcine alveolar macrophages (PAMs) followed by two successive *in vivo* passages in pigs. The animals used for providing PAMs and for virus adaptation were obtained from the same farm, treated and reared under the same conditions, as mentioned above. Piglets used for *in vivo* passages received 10 mL of the tissue culture supernatant adjusted to 10^6 $TCID_{50}$/mL via intra tracheal inoculation. On the seventh day post-inoculation (p.i.), when respiratory distress became evident, the piglets were euthanised and their lungs were aseptically collected and homogenized in RPMI medium supplemented with 200 U/mL of penicillin, 200 µg/mL of streptomycin and 50 µg/mL gentamycin. Each challenged piglet received 10 mL of clarified 5% lung homogenate in sterile RPMI via intra- tracheal injection, corresponding to an estimated dose of $5x10^5$ $TCID_{50}$ of virus, as established by back titration. The inoculum was further tested for the presence of the above indicated porcine pathogens and no agent other than PRRSV could be isolated.

3.3.3 Plasmids and recombinant proteins

Viral RNA was extracted from PRRSV-infected MARC-145 cells by the guanidinium isothiocyanate-acid phenol method (Chomczynsky & Sacchi, 1987). The ORF5 encoding region was amplified by RT-PCR, cloned in pGEX-4T1 plasmid (Pharmacia) and a recombinant fusion protein consisting of glutathione sulfotransferase (GST) joined to the N-terminus of the ORF5 protein (GST-ORF5) was expressed in *E. coli* and purified by affinity chromatography on glutathione Sepharose column as previously described (Pirzadeh & Dea, 1997). Subsequent SDS-PAGE analysis of the purified protein confirmed that no contaminating bacterial proteins were present in the purified recombinant fusion protein (data not shown). Plasmidic DNA was purified from bacterial lysates by anion exchange chromatography on a hydroxyapatite column (QIAGEN Inc, Chatsworth, CA) and then precipitated by isopropanol. This procedure effectively eliminated bacterial protein contaminants. The same amplification product was cloned in pET 21a plasmid (Novagen) to produce a recombinant protein in *E. coli* consisting of the ORF5-encoded protein fused at it's C-terminus to 6 histidine residues (ORF5-pH). The ORF5 coding region was further cloned into the *Hind* III and *Xba* I cloning sites of the eukaryotic expression vector pRc/CMV (Invitrogen), down-stream of the human cytomegalovirus (HCMV) promoter, to produce pRc/CMV5. The sequence of the oligonucleotide primers used for the latter amplification were as follows:ETS 5 (forward primer) : 5'- AAG CTT GCC GCC GCC ATG TTG GGG AAA TGC TTG ACC- 3', which comprises the first ATG codon of the ORF5 gene downstream of a Kozak motif for initiation of translation in vertebrates (Kozak, 1987), and ETR5 (reverse primers): 5'- TCTAGAGGCAAAAGTCATCTAGGG-3', which comprises the 3' stop codon of the viral gene. The nucleotide sequence accession number (EMBL/GenBank/DDBJ libraries) of IAF-Klop strain is U64928. For directional cloning, *Hind* III and *Xba* I restriction sites were added at the 5' ends of the sense and antisense oligonucleotide primers, respectively. Both strands of pRc/CMV5 were sequenced by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using the T7 DNA polymerase (Pharmacia Biotech Inc.) in

an Automated Laser Fluorescent DNA sequencer (Pharmacia LKB) in order to confirm that no error has occurred as a result of PCR amplification.

3.3.4 Transient expression of the GP_5 glycoprotein

Ex vivo expression of pRc/CMV5 construct was tested in transient expression experiments in COS7 and 293 cells maintained as confluent monolayers. Cells in 6 cm-tissue culture plates were transfected with 15 μg of plasmid DNA by calcium phosphate co-precipitation (Graham & van der Eb, 1973). For indirect immunofluorescence (IIF), cells were incubated at 37° C and fixed with 80% cold acetone for 20 min at 4° C at various times (18 to 72 h) post-transfection. The monolayers were then reacted for 30 min with anti-ORF5 rabbit monospecific hyper-immune serum (Mardassi *et al.*, 1996) and the immune reaction was revealed following incubation with fluorescein-conjugated goat anti-rabbit Ig (Boehringer Menheim), as previously described (Loemba *et al.*, 1996).

3.3.5 Mice immunization schedule

In vivo expression of pRc/CMV5 was verified by immunizing groups of five CD-1 or BALB/c mice (G1) with 50μg of pRc/CMV5 diluted in 50 μL of phosphate buffered saline (PBS), and injected in the *tibialis cranialis* muscle with a 27 gauge needle. The mice were boosted twice with the same quantities of DNA at two week-intervals. Control mice (G3) received the same amounts of parental pRc/CMV vector via identical route and frequency. G2 mice received three doses of 50μg of GST-ORF5 in Freund's complete or incomplete adjuvant intraperitoneally.

3.3.6 Pig immunization schedule

Piglets in group G1 were injected with 100μg of pRc/CMV5 diluted in 0.5 mL of PBS. Two-third of the volume was injected using a 26 gauge needle in the

tibialis cranialis muscle of the right and one-third was intradermally administered into the dorsal surface of the ear. Control piglets in group G3 and G4 received 100μg of the parental vector via identical route and frequency. Pigs in group G2 received via intramuscular injection 300μg of GST-ORF5 in complete or incomplete Freund's adjuvant. Immunization frenquency and intervals are mentioned in Table 4.1.

3.3.7 Virus neutralization and serological tests

Mice and pig sera were tested for the presence of specific anti-GP_5 antibodies by virus neutralization (VN), IIF, ELISA and Western immunoblotting (WB) tests. The VN test was performed in triplicates with 100 μL of serial dilutions of heat-inactivated (56° C, 45 min) test sera, incubated for 60 min at 37° C in the presence of 100 $TCID_{50}$ of the virus in DMEM, the mixtures were put in contact with confluent monolayers of MARC-145 cells seeded in 96 well-microtitration plates 48-72 h earlier. Cell monolayers were incubated at 37° C in a humidified atmosphere containing 5% CO_2, and observed daily for up to five days for the appearance of cytopathic effects (CPE). The monolayers were then fixed with a solution of 80% methanol containing 0,05% H_2O_2 and tested for expression of the PRRSV N protein by IPMA (Wensvoort *et al.*, 1991b), using the N protein specific MAb IAF-K8 (Dea *et al.*, 1992b). Plates were subsequently incubated with peroxidase-labelled goat anti-mouse IgG (Boehringer Mannheim). Neutralizing titres were expressed as the reciprocal of the highest dilution which completely inhibited the expression of viral N protein. IIF was performed on PRRSV-infected and acetone-fixed MARC-145 cells, as previously described (Loemba *et al.*, 1996). Indirect ELISA was essentially performed as previously described (Dea & Tijssen, 1989) with minor modifications. Gel-purified ORF5-pH protein (0.1 μg of protein/well) in 0.05M-sodium carbonate buffer, pH 9.6, was used to coat flat-bottomed microtitration plates and peroxidase-labelled goat anti-porcine IgG was

used to detect the captured antibodies. The substrate solution consisted of 0.1% urea peroxide and 0.02% 3,3',5,5'-tetramethyl benzidine, in 10 mM citrate buffer, pH 5.0, mixed in equal volumes, and the absorbance values were determined at 450 nm. WB was also performed as previously described (Pirzadeh & Dea, 1997) using either ORF5-pH protein or sucrose gradient purified-PRRSV as antigen.

3.3.8 Blastogenic transformation test

At regular post-immunization intervals, pigs were medicated with Xylazine (Bayers) at a dose of 1mg/Kg and blood samples were collected from the anterior *vena cava* in vacuum tubes containing 1/10 volume 150 mM sodium citrate in PBS, and then diluted 1:3 in sterile RPMI. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Paque (density 1.077, Pharmacia) centrifugation at 1,200xg for 20 min. The mononuclear cells were collected from the buffy coat, pelleted and the residual red blood cells were lysed by incubating cells with 0.53% ammonium chloride for 10 min at 37°C. After two washes in RPMI, the leukocyte suspension was adjusted to a suspension of 2 x 10^6 cells per mL in RPMI containing 20% homologous heat inactivated PRRSV negative porcine serum, 50 U/mL of penicillin and 50 μg/mL of streptomycin. The antigen-specific proliferation was determined by incubating PBMC in microtitration plates (4 X 10^5 cells in 200μL/well in triplicate) for 72 h in presence of various concentrations (0, 0.1, 10 and 25μg/ml) of ORF5-pH protein. Blastogenic capacity of the PBMC under test conditions was confirmed by including control in triplicate containing 2.5, 5 or 10 μg/mL of concanavaline A (ConA, Sigma Chemicals). After a 72 h stimulation period, the cells were labelled for 18 h with 0.1 μCi of [^{3}H]thymidine (Amersham) per well, harvested with a semiautomatic cell harvester (Skatron Instruments) and the incorporated radiolabelled nucleotide was measured by scintillation counting after addition of a fluorescent liquid scintillator (Cytoscint, ICN). The level of proliferation was expressed as the mean of counts per minute (CPM) of the test wells minus the mean of the background CPM in control wells. The control for background levels consisted of PBMC cultures in medium alone.

3.3.9 Virus isolation

After collection of blood samples, pigs were euthanised by rapid intravenous injection of sodium pentobarbital (MTC Pharmaceuticals). Specimens were aseptically collected from lungs, spleen, liver, kidneys, mediastinal and mesenteric lymph nodes, then tissue homogenates were prepared in DMEM to final concentrations of 1:20 and 1:100. Following clarification by centrifugation at 10,000 g for 10 min, tissue homogenates were inoculated onto monolayers of MARC-145 cells in 24 well-culture plates or PAMs seeded in 96 well-microtitration plates. Cells were harvested by 2 freeze-thaw cycles at 4-5 days post-inoculation, tissue culture supernatants were clarified and used for a second passage. Cultures were observed daily for CPE until day five post-inoculation, then infected monolayers were fixed with cold acetone for IIF.

3.3.10 Detection of viral genome by RT-PCR

Total RNA was extracted from tissues collected from challenged animals and from MARC-145 cells inoculated with tissue homogenates. RT-PCR was performed using oligonucleotide primers 1006PS + 1007PR and 1008PS + 1009PR to amplify ORF6 and ORF7 genomic regions of PRRSV respectively , as previously described (Mardassi *et al.*, 1995).

3.3.11 Histopathological examination

Thin sections (5 μm thick) of formalin-fixed, paraffin embedded tissues from the lungs, spleen, liver, kidneys, thoracic and mesenteric lymph nodes of all pigs were routinely processed for the hematoxylin-phloxin-safran (HPS) staining, as described previously (Dea *et al.*, 1991).

3.4 Results

3.4.1 Transient expression of cloned ORF5 gene

Expression of the ORF5 product was demonstrated in both COS7 and 293 cells lines at 24 and 36 h post-transfection. The identification of GP_5 was confirmed by IIF using monospecific anti-ORF5 rabbit antiserum or the porcine anti-PRRSV serum. As shown in Fig. 4.1, an intense cytoplasmic fluorescence could be observed in approximately 10 to 15% of the cells, and the expressed GP_5 tended to accumulate near the perinuclear region.

3.4.2 Antibody response of mice and pigs

Sera collected at various times post-immunization (Table 4.1) were positive for the presence of anti-PRRSV antibodies by IIF. The protein specificity of mice and pigs sera to GP_5 was established by immunoblotting with purified whole virus and *E. coli*- expressed recombinant ORF5-pH fusion protein (Fig 4.2) and by ELISA (Table 4.1). BALB/c mice inoculated with the GST-ORF5 or pRc/CMV5 developed neutralizing antibodies which could be first detected two weeks after the second booster injection. The VN titres of BALB/c mice sera were estimated between 32 and 64 by the 8th week post-immunization and persisted through the end of the 12 week-observation period. In contrast, the CD-1 mice did not developed neutralizing antibodies to PRRSV despite a significant anti-ORF5 specific antibody response, detected by ELISA and IIF. Seroconversion was also demonstrated by IIF and ELISA in both groups of immunized pigs (Table 4.1) 15 days after first injection of either GST-ORF5 or pRc/CMV5. Neutralizing antibodies were detected in sera of the DNA-immunized pigs only two to three weeks after the second booster injection (eight to nine weeks after first inoculation of plasmidic DNA), and two weeks after the PRRSV challenge with estimated titres close to 128. None of the virus challenged animals in the unvaccinated or GST-ORF5 immunized group (G2 and G3) developed detectable neutralizing antibodies

(VN titres < 8) to PRRSV two weeks after infection (Table 4.1). Unvaccinated-unchallanged control animals in group G4 tested negative to PRRSV and ORF5-pH protein as determined by IIF and ELISA throughout the observation period.

3.4.3 Specific blastogenic response to ORF5-pH

PBMCs obtained from both groups of immunized pigs underwent specific blastogenic transformation *ex vivo* in a dose dependent manner in the presence of ORF5-pH protein, whereas [^{3}H]thymidine incorporation of the PBMCs obtained from unvaccinated animals remained at basal level (Fig 4.3). Blastogenic transformation indexes of 7-12 and 10-12 were calculated two weeks after the second booster injection of GST-ORF5 and pRC/CMV5, respectively. Concentrations higher than 10 μg of the ORF5-pH protein per mL of culture medium did not increase [^{3}H]thymidine incorporation levels in PBMCs from both groups of pigs. No significant variations ($t > 0.05$) were observed in blastogenic response to ConA of vaccinated pigs compared to unvaccinated controls (Fig. 4.4).

3.4.4 Clinical observations

Unvaccinated virus challanged pigs (G3) developed clinical signs of respiratory disease, beginning two to three days after virus challenge, which persisted through the end of the two week-observation period. The principal signs included a marked drop in feed consumption, hyperthermia (40.2 to 41.7) that persisted for 10 to 14 days, eyelid oedema, laboured breathing (abdominal respiration) in two pigs accompanied by rasping and crowing sounds heard during inspiration. Apart from a transitory mild fever (39.8-40.4°C) that lasted not more than two to three days, all vaccinated pigs remained clinically healthy during the two week observation period following virus challenge. Their average feed consumption and growth rate remained identical to those of unvaccinated unchallenged controls.

3.4.5 Virus isolation

As summarized in Table 4.2, after a single passage on MARC-145 cells, virus was recovered from tissue homogenates (dilutions 1/20 and 1/100) of several organs (lungs, spleen, kidneys, liver, lymph nodes) of unvaccinated animals (G3) two weeks after virus challenge, whereas apart from lungs and mediastinal lymph nodes no virus was isolated from other organs of DNA immunized pigs after two successive passages, indicating the generalized viremia of unvaccinated pigs compared to respiratory tract localization of virus in DNA immunized animals. PRRSV was also recovered from spleen and kidneys of one of the three GST-ORF5 immunized pigs. Presence of the viral genome in lungs of all three groups of animals could be demonstrated by RT-PCR, but only in the spleen of unvaccinated challenged pigs and those which have been immunized with GST-ORF5. Furthermore, PRRSV burden was lower in lungs and mediastinal lymph nodes of DNA vaccinated pigs since it could not be recovered after two passages on MARC-145 cells from the 1/100 dilution of tissue homogenate. The presence of viral antigen could only be detected in the 1/20 dilution of lung and mediastinal lymph nodes homogenates, with delayed appearance of CPEs suggestive of low virus titres.

3.4.6 Necropsy findings

Unvaccinated, virus-challenged pigs (G3) euthanised at day 14 post-inoculation had gross lesions that were confined to the respiratory tract and thoracic cavity. Portions of the lungs were tan and partly collapsed, with occasional anteroventral areas of congestion and consolidation. The mediastinal lymph nodes were enlarged and congested. Adherence of the pleura to the thoracic cage was observed in one pig, with slight accumulation of non-suppurative exudate within the thoracic cavity (hydrothorax) and pericardium (hydropericardium). No significant gross lesions were observed in the other organs. Pulmonary hepatisation and glandular aspect at lung section was remarkable in one of the GST-ORF5-immunized animals. Apart

from mild tumefaction of mediastinal lymph nodes in one of the DNA vaccinated pigs, no significant gross abnormalities were observed in this group of animals. Microscopic lesions observed in unvaccinated, virus-challenged pigs were confined to the lungs and consisted of macrophage infiltration, pyknotic cell debris and protein-rich exudate in the lumen of large bronchi and bronchioli, a peribronchiolar and perivascular lymphomononuclear cell infiltration, the presence of lymphomononuclear cells within the alveolar lumen with hyperplasia of type II pneumocytes, mononuclear cells invasion and the presence of pyknotic cells in alveolar septae (Fig. 4.5 b, c, and d). The GST-ORF5 immunized pigs developed intense interstitial pneumonitis, characterized by hyperplasia of bronchiolar epithelium and pneumocytes type II of the alveolar endothelium, perivascular cuffing, lymphomononuclear cells infiltration and thickening of alveolar septae (Fig. 4.6 a and b). A remarkably milder interstitial pneumonitis was observed in the DNA vaccinated pigs. In those pigs, large airways (bronchi, bronchioli), as well as alveolar ducts, were normal in appearance with absence of cells and cellular debris within the lumen (Fig. 4.6 c and d).

3.5 Discussion

In vivo expression of foreign proteins via simple injection of plasmidic DNA into mammals was first described in 1980s (Benvenisty & Reshef, 1986; Desrosiers *et al.*, 1985; Nicolau *et al.*, 1983; Seeger *et al.*, 1984; Wang *et al.*, 1987; Wolff *et al.*, 1987) and few years later *in vivo* gene transfer by microprojectiles and particle bombardment was studied (Sanders Williams *et al.*, 1991; Yang *et al.*, 1990). Encouraging results have been also obtained by DNA immunization against viral pathogens and with few exceptions, laboratory animals such as mice (Davis *et al.*, 1994; Martins *et al.*, 1995; Kuhöber *et al.*, 1996; Ulmer *et al.*, 1993; Yokoyama *et al.*, 1995) rat (Benvenisty & Reshef, 1986) or guinea pigs (Bourne *et al.*, 1996) and rabbits (Sundaram *et al.*, 1996) have been generally used as the experimental animals. Only a few experiments have been reported on DNA immunization in the literature in farm animals including cattle (Cox *et al.*, 1993),

horses (Lunn *et al.*, 1996), chickens (Fynan *et al.*, 1996; Robinson *et al.*, 1993), and more recently pigs (Gerdst et al., 1997). Our poor knowledge of the histocompatibility system of farm animals and the difficulties in handling large animals may be discouraging for some investigators.

In this report, DNA immunization was used to evaluate the immunogenicity of a single gene product of a viral pathogen in its natural host. The data obtained demonstrate that the ORF5-encoded GP_5 of PRRSV elicits neutralizing antibodies in pigs. Furthermore, these experiments suggest that conformational neutralizing epitopes may also be associated with the GP_5 of PRRSV, since immunizing pigs with the recombinant GST-ORF5 protein failed to trigger the immune system to produce neutralizing antibodies. Indeed, fusion proteins expressed in prokaryotic vectors may not effectively mimic the native viral proteins due to the differences in post-translational modifications notably polypeptide folding, disulfide-bonds formation and N-glycosylation. It can therefore be assumed that the induction of neutralizing antibodies in DNA-immunized animals is due to correct glycosylation and post-translational modification of the GP_5 expressed in host cells. Recently, recombinant adenoviruses carrying the ORF5 gene of IAF-Klop strain of PRRSV, downstream of the CMV promotor were constructed and the ORF5 product expressed in human 293 cells was found to be a glycosylated protein (Gagnon *et al.*, 1997). The development of neutralizing antibodies cannot be attributed to the virus challenge since they were detected in sera of DNA-immunized pigs only and at 14 days post-challenge, unvaccinated control pigs had no detectable neutralizing antibodies. Furthermore, previous studies demonstrated that neutralizing antibodies to PRRSV in naturally or experimentally infected pigs start to be detected at low titres as late as three to four weeks after exposure to virus (Loemba *et al.*, 1996; Nelson *et al.*, 1994; Yoon *et al.*, 1995), whereas in the present study neutralizing antibodies were detected in sera of DNA immunized pigs two to three weeks after the second booster injection and significant titres of 64 to 128 were detected 14 days after virus challenge. This period corresponded to the minimum time lapse necessary for pigs to develop circulating antibodies following natural or

experimental exposure to PRRSV (Joo *et al.*, 1997; Loemba *et al.*, 1995; Wills *et al.*, 1997b). The appearance of neutralizing antibodies in sera of DNA-immunized, PRRSV-challenged pigs may suggest that virus challenge had a booster effect in developing neutralizing antibodies. This is unlikely since BALB/c mice also developed neutralizing antibodies in the absence of PRRSV challenge.

Neutralizing antibodies in GST-ORF5 immunized BALB/c mice can be attributed to relatively high quantities of antigen used for immunizing mice compared to that used in pigs (50 μgper mouse versus 300 μg per pig). An alternative interpretation of this part of the results may be that CD-1 mice and pigs are tolerant to the linear epitopes of the *E. coli*-expressed ORF5-encoded recombinant protein, since CD-1 mice and pigs immunized with GST-ORF5 failed to develop neutralizing antibodies to PRRSV (Table 4.1). The lower titres of anti-GP_5 antibodies in DNA-immunized mice and pigs compared to the GST-ORF5 immunized animals can be explained by the fact that the injected antigens are available to the B cells and other antigen presenting cells which can potentially stimulate a strong antibody response. On the contrary, the expressed protein may be retained within the DNA recipient cells and released in rather smaller amounts within the extracellular space, either by secretion or cell death as a result of cytotoxic protein accumulation. It was previously established that the GP_5 of PRRSV has an apoptotic effect on transfected cells (Suárez *et al.*, 1996a). Previous studies indicate that most proteins eliciting high antibody response after DNA immunization are membrane-associated or secreted proteins (Bourne *et al.*, 1996; Davis *et al.*, 1994; Fynan *et al.*, 1993; Robinson *et al.*, 1993; Ulmer *et al.*, 1993). While GP_5 is a membrane-associated viral glycoprotein, it retains a perinuclear location within the mammalian cells in transient expression experiments (Fig.4.1).

Previous studies have established that protection due to DNA immunization is mediated by both $CD4^+$ and $CD8^+$ T lymphocytes (Doolan *et al,* 1996; Manickan *et al.*, 1995). According to experimental data on mice, the type of immune

response also depends on route of DNA immunization. It has been previously demonstrated that intradermal immunization can induce very high levels of cytotoxic T lymphocytes mediated by Th2 like response, whereas intramucular DNA inoculation favors a Th1 like response (Pertner *et al.*, 1996; Yokoyama *et al.*, 1997). Since such data is not yet available for large animal models, we used the two inoculation routes simultaneously in order to enhance both types of immune response. In the present study, proliferation assays of PBMCs from pRc/CMV5-and GST-ORF5-immunized pigs, showed a specific blastogenesis following stimulation by the recombinant ORF5-pH protein, independent from production of specific anti-PRRSV neutralizing antibodies. Since the identity of PBMCs subpopulation(s) which underwent blastogenesis following antigenic stimulation was not determined, it can be suggested that antigen-specific proliferation may be due to $CD4^+$ and B cell effector cells implicated in eliciting an antibody response.

Our results showed that DNA immunization with a plasmid encoding the GP_5 of PRRSV protected pigs from developing intensive PRRSV-induced lesions observed in unvaccinated virus-challenged controls. Virus dissemination to organs other than the lungs and the accessory lymph nodes was not observed in DNA-vaccinated animals even after a massive virus challenge, and these animals had a remarkably lower virus burden in their respiratory system compared to the GST-ORF5 immunized or unvaccinated controls. Therefore it appears that ORF5 may be a good candidate for a subunit recombinant-type vaccine against PRRSV. However, the genomic variabilities of the ORF5 genes which have been recently reported amongst North American field isolates (Meng *et al.*, 1995; Pirzadeh *et al.*, 1997) and between North American and European strains (Mardassi *et al.*, 1995; Murtaugh *et al.*, 1996; Suarez *et al.*, 1996), need further investigations in order to determine their significance in term of antigenic determinants involved in protection.

As expected, DNA immunization was not sufficient to inhibit virus persistence and shedding in the respiratory tract during the short 14 day observation period

after virus challenge. However, considering the high callenge dose used in our experiments, the protection conferred to the DNA-vaccinated pigs was quite remarkable. Other investigators have reported similar results in connection with post-challenge virus persistence in DNA-immunized animals (Bourne *et al.*, 1996; Cox *et al.*, 1993; Martins *et al.*, 1995; Ulmer *et al.*, 1993). Mucosal immunity is believed to play a role in protection against PRRSV infection, virus persistence and shedding but this aspect of immunity against PRRSV has not been investigated so far.

Finally, the intensive interstitial pneumonia observed in GST-ORF5 immunized animals could be attributed to the antibody-dependent enhancement (ADE) phenomenon which has been reported to occur in PRRSV-infected pigs (Yoon *et al.*, 1996). According to the results obtained by these investigators, subneutralizing levels of anti-PRRSV antibodies have the potential to intensify pathogenesis of PRRSV infection due to ADE, but virus replication is significantly inhibited in presence of neutralizing antibody titres. The immunoglobulins used in passive immunization studies to demonstrate the occurrence of ADE in PRRSV-infected pigs were extracted from polyclonal pig convalescent sera, but results from immunoblotting studies suggested that the ADE was mediated by antibodies specific to the GP_5 (Yoon *et al.*, 1996). The intensity of the lymphomononuclear cells infiltration observed in the lungs of GST-ORF5 immunized pigs which failed to develop neutralizing antibodies to the GP_5, may also suggest that subneutralizing anti-GP_5 antibodies contribute to ADE of PRRSV infection or alternatively, the enhanced local secretion of inflammatory cytokines in this group of animals.

* Groups of 5 mice or 3 piglets were immunized by pRc/CMV5 plasmid or GST-ORF5 expressed in E. Coli on the mentioned days. Blood samples were collected from retro-orbital vein of mice or anterior vena cava of pigs prior to each immunization.

† Sample collection only.

‡ Pigs were challenged with 5 ´ 105 TCID50 by intra-tracheal inoculation.

§ Control animals consisted of 5 BALB/c mice, 5 CD-1 mice and 2 x 3 F1 piglets (G3 and G4). Each control animal was injected with corresponding quantities of parental pRc/CMV plasmid via identical route and frequency. Control mice and G4 control pigs (unvaccinated-unchallenged) remained sero-negative throughout the observation period.

ELISA: Reciprocal of highest serum dilution reacting with the recombinant ORF5-pH expressed in E. Coli.

IIF: Reciprocal of highest serum dilution at which specific cytoplasmic fluorescence was observed in PRRSV-infected MARC- 145 cells.

VN: Reciprocal of highest serum dilution which inhibited 100% of CPE and expression of N viral protein in PRRSV (IAF-Klop strain) infected MARC 145 cells stained by IPMA.

Antibody titres correspond to the average titres ± standard deviation.

Table 4.1 Antibody response of DNA and GST-ORF5 immunized mice and pigs.

Animal Group	Immunogen and dose	Serological tests	Immunization and sample collection schedules (days)				
			0 *	15 *	37 *	51 †‡	65 †
CD-1 mice §	G1: 50μg pRc/CMV5	ELISA	–	140 ± 49	560 ± 196	640 ± 196	640 ± 196
		IIF	–	35 ± 16	51 ± 16	51 ± 16	51 ± 16
		VN	–	–	–	–	–
	G1: 50μg GST-ORF5	ELISA	–	320 ± 98	8,960 ± 3,135	10,240 ± 3,135	> 12,800
		IIF	–	29 ± 6	45 ± 16	102 ± 31	102 ± 31
		VN	–	–	–	–	–
BALB/c mice §	G1: 50μg pRc/CMV5	ELISA	–	260 ± 120	560 ± 196	560 ± 196	960 ± 196
		IIF	–	22 ± 8	45 ± 16	102 ± 31	102 ± 31
		VN	–	–	< 8	51 ± 37	58 ± 31
	G2: 50μg GST-ORF5	ELISA	–	640 ± 196	2,560 ± 784	4,800 ± 2,024	2,560 ± 784
		IIF	–	102 ± 31	320 ± 98	640 ± 196	480 ± 160
		VN	–	–	< 8	45 ± 16	102 ± 31
Pigs §	G1: 100μg pRc/CMV5	ELISA	–	133 ± 47	533 ± 189	667 ± 189	667 ± 189
		IIF	–	64 ± 0	107 ± 30	533 ± 189	667 ± 189
		VN	–	–	< 8	< 8	107 ± 30
	G2: 300μg GST-ORF5	ELISA	–	400 ± 0	4,267 ± 1,508	> 12,800	> 12,800
		IIF	–	107 ± 30	1,333 ± 377	667 ± 189	667 ± 189
		VN	–	–	< 8	< 8	< 8

Table 4.2. Virus isolation and RT-PCR analysis performed on day 14 post-challenge.

Pig immunized with:	Virus isolation on MARC-145 cells										RT-PCR			
	Organ						Lymph Nodes							
	Lung		Spleen		Kidney		Liver		Mediastinal		Mesenteric		Lung		Spleen	
	First Passage															
GST-ORF5	+	(3)	+	(1)	-	(0)	-	(0)	-	(3)	-	(0)	+	(3)	+	(2)
pRc/CMV5	-	(0)	-	(0)	-	(0)	-	(0)	-	(0)	-	(0)	+	(3)	-	(0)
Unvaccinated controls	++	(3)	++	(3)	++	(3)	++	(3)	++	(3)	-	(0)	+	(3)	+	(3)
	Second Passage															
GST-ORF5	++	(3)	+	(1)	+	(1)	-	(0)	+	(3)	-	(0)				
pRc/CMV5	+	(3)	-	(0)	-	(0)	-	(0)	+	(1)	-	(0)				
Unvaccinated controls	++	(3)	++	(3)	++	(3)	++	(3)	++	(3)	-	(0)				

+: Virus could be recovered from $^{1}/_{20}$ dilution of organ homogenates.

++: Virus could be recovered from $^{1}/_{100}$ dilution of organ homogenates.

Of the three pigs studied in each case, the number that tested positive is indicated in parentheses.

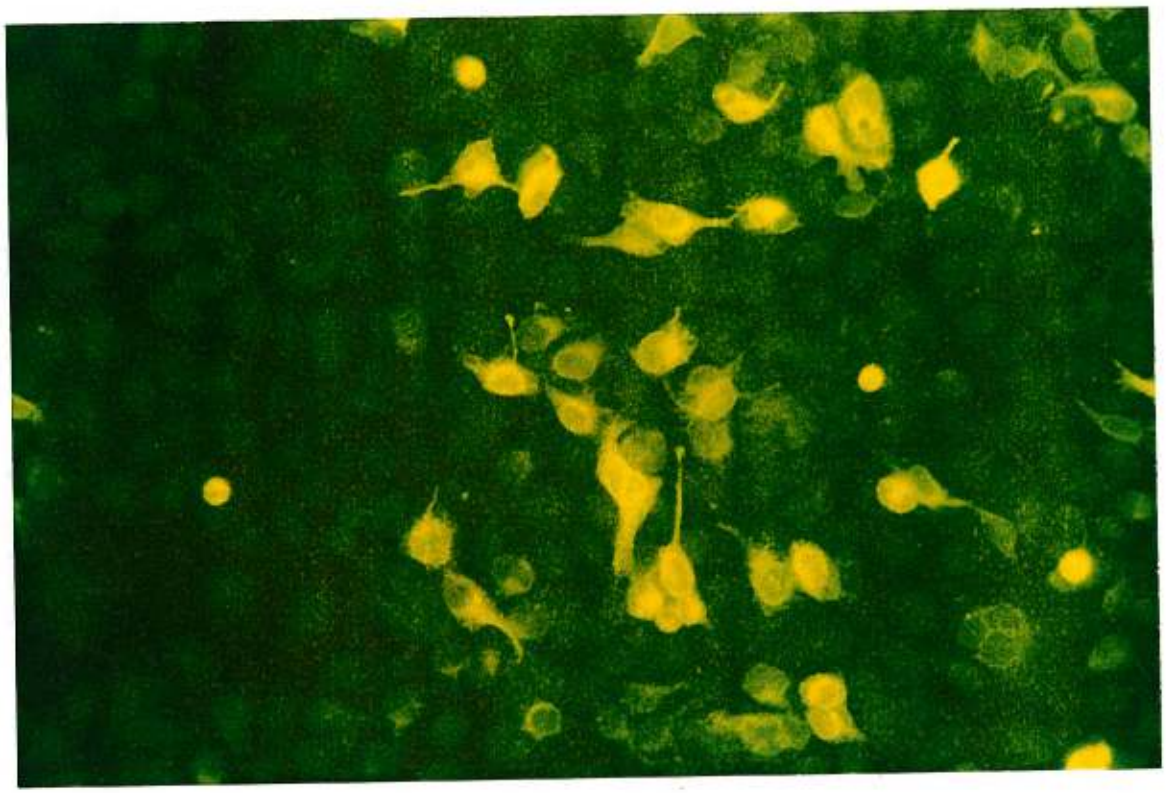


Figure 4.1

Immunofluorescent staining of COS7 cells at 24h post-transfection with pRc/CMV5 plasmid. Expression of GP_5 of PRRSV (IAF-Klop strain) was confirmed by IIF following incubation in the presence of the rabbit anti-ORF5 monospecific serum. A similar fluorescent profile was obtained following incubation with the autologous anti-PRRSV porcine hyperimmune serum. Expressed GP_5 protein mostly accumulated in the perinuclear region.

Figure 4.2

Reactivity by immunoblotting of the serum of DNA-immunized pigs and mice on day 51 post-immunization towards the GP_5 of PRRSV and the recombinant ORF5-pH protein expressed in *E. coli*. Lane 1: Immunoblot showing reactivity of a convalescent pig serum towards three major structural proteins (N, M and GP_5) of PRRSV (IAF-Klop strain). Lanes 2: Reactivity of pRc/CMV5-immunized pig serum towards GP_5 of PRRSV. Lane 3: reactivity of pRc/CMV5 immunized mouse serum with GP_5 of PRRSV. Lanes 4: reactivity of pRc/CMV5 immunized pig serum with ORF5-pH recombinant protein. Lane 5: reactivity of pRc/CMV5 immunized mouse serum with ORF5-pH recombinant protein. Lane 6: reactivity of porcine convalescent serum with ORF5-pH recombinant protein.

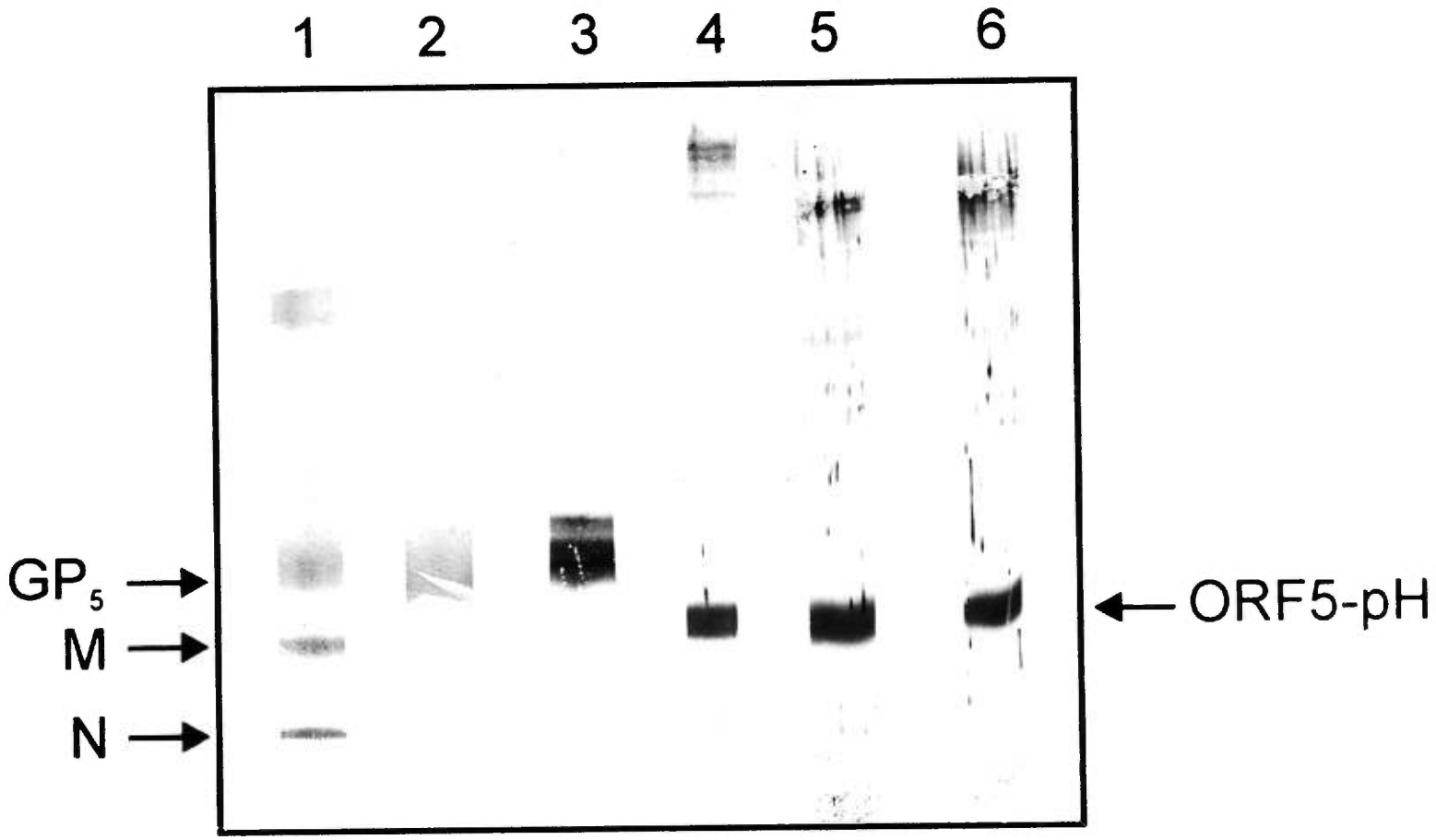

1
2
3
4
5
6
GP5
M
N
ORF5-pH

Figure 4.3

Ex vivo blastogenic response of porcine PBMCs following incubation in presence of different concentrations of ORF5-pH recombinant protein at variable times post-immunization. PBMCs obtained from both GST-ORF5 or pRc-CMV5 immunized pigs underwent blastogenesis in presence of ORF5-pH and stimulation indexes of 7 to 12 were calculated.

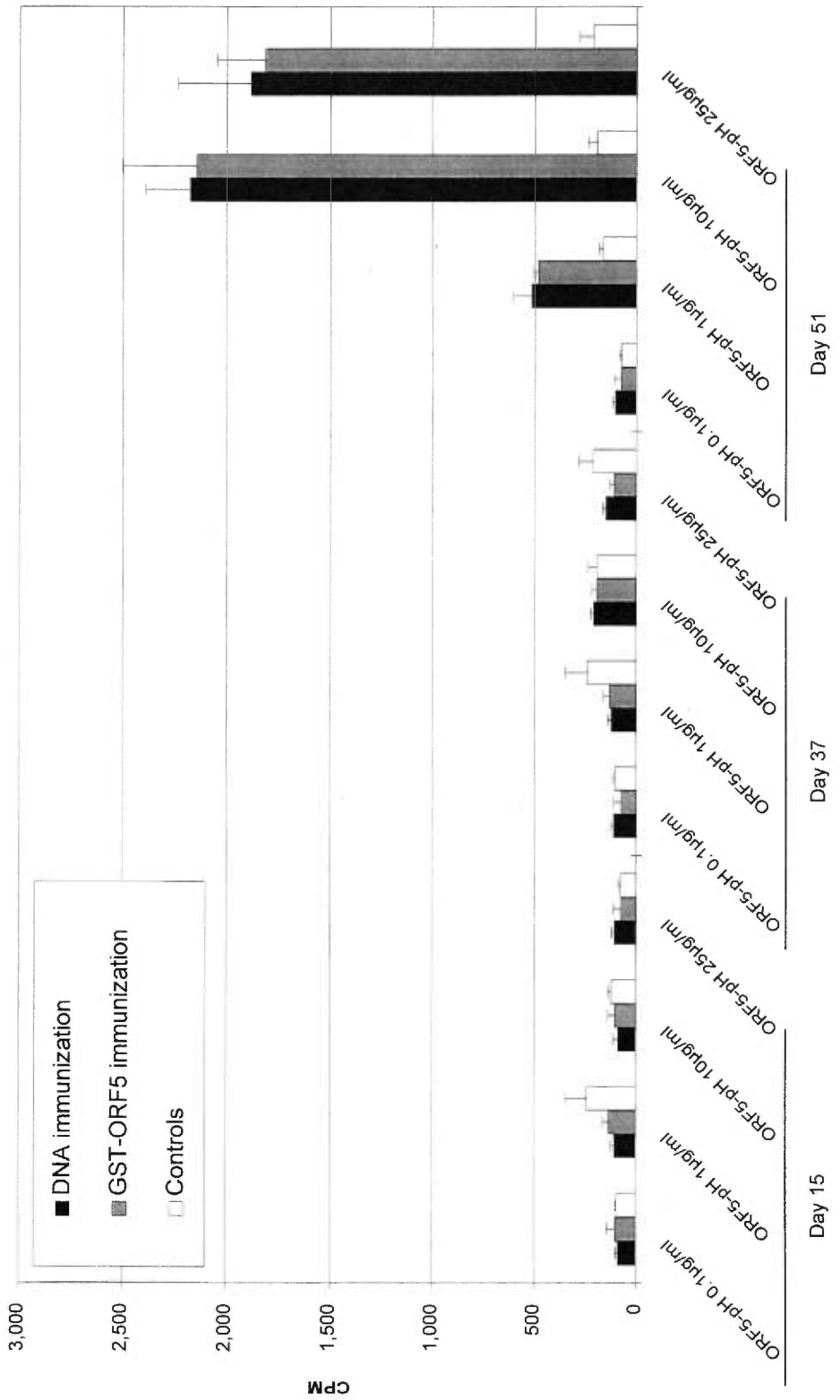

DNA immunization
GST-ORF5 immunization
Controls
CPM
3,000
2,500
2,000
1,500
1,000
500
0
ORF5-pH 0.1ug/ml
ORF5-pH 1ug/ml
ORF5-pH 10ug/ml
ORF5-pH 25ug/ml
Day 15
Day 37
Day 51

Figure 4.4

Ex vivo non-specific blastogenic response of porcine PBMCs following incubation in the presence of different concentrations of concanavaline A at variable times post-immunization. No significant difference was observed in non-specific mitogen induced blastogenesis response of PBMCs obtained from GST-ORF5 or pRc/CMV5 immunized and unvaccinated pigs at any time after immunization or virus challenge ($p > 0.05$).

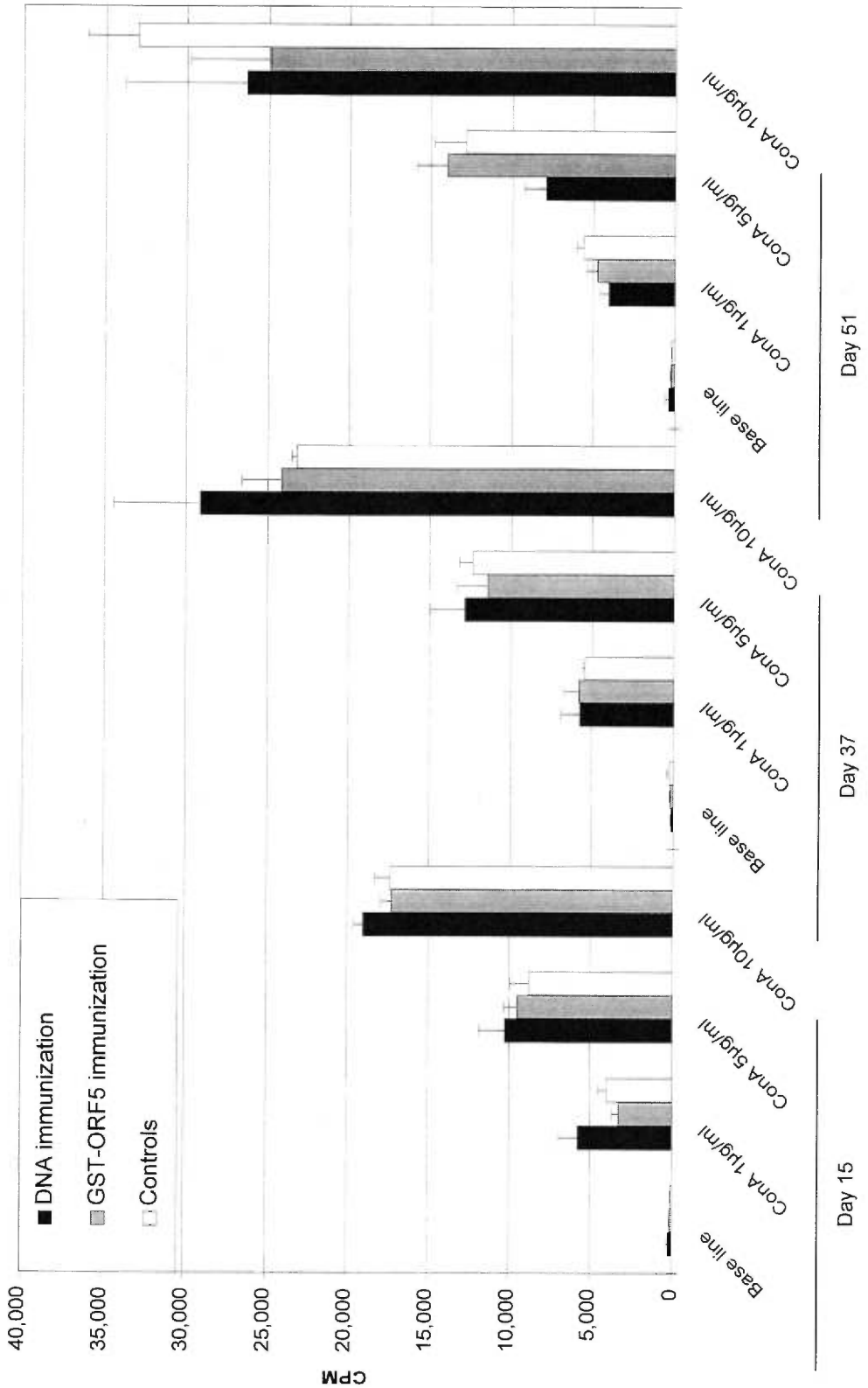

DNA immunization
GST-ORF5 immunization
Controls
CPM
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
Base line
Con A 1ug/ml
Con A 5ug/ml
Con A 10ug/ml
Day 15
Base line
Con A 1ug/ml
Con A 5ug/ml
Con A 10ug/ml
Day 37
Base line
Con A 1ug/ml
Con A 5ug/ml
Con A 10ug/ml
Day 51

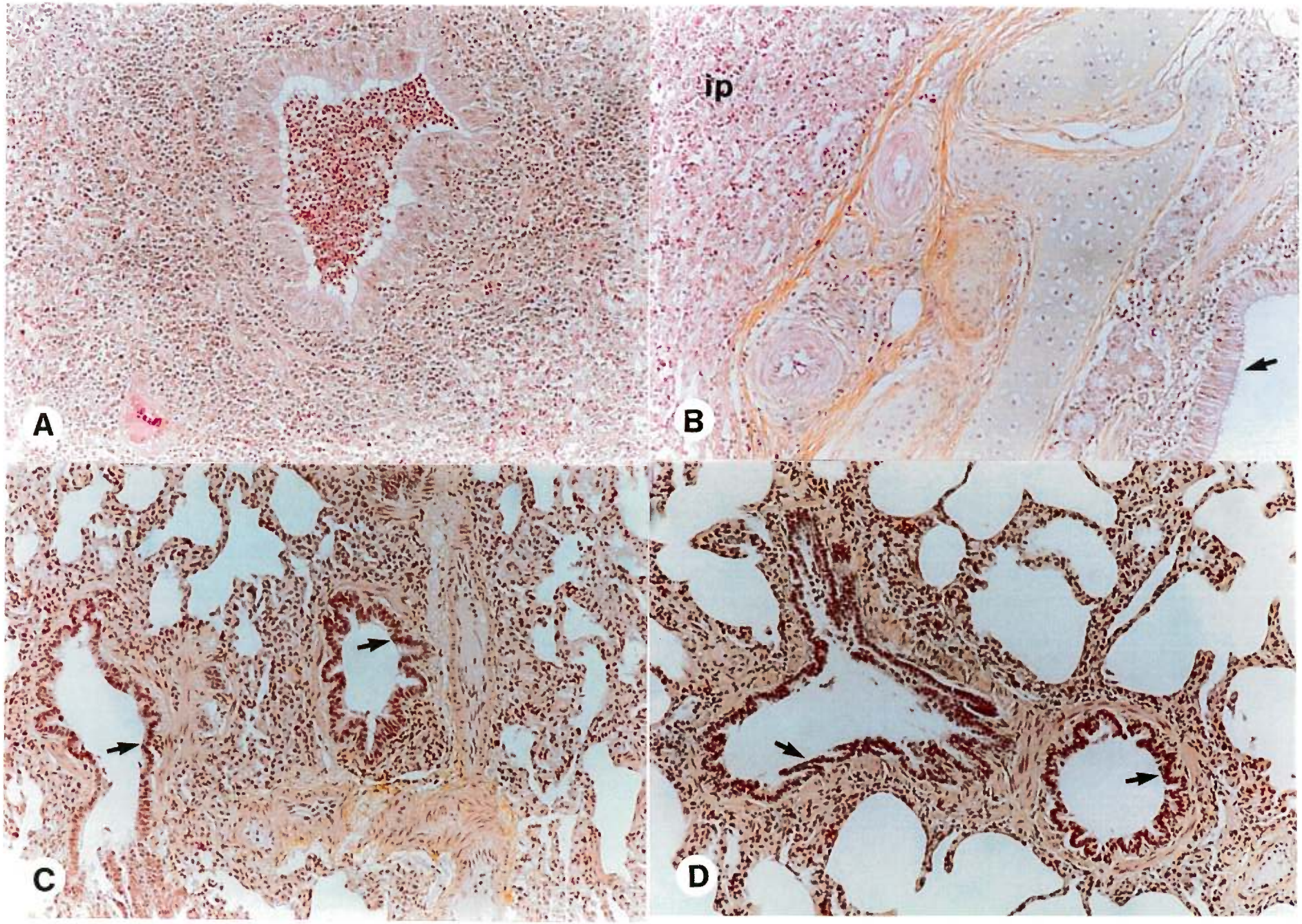
Figure 4.5

Histological findings in lungs of control (a) and unvaccinated PRRSV-challenged (b,c,d) pigs. (a) Spongiform aspect of the lung of a normal pig showing clear airway passages (bronchiole and alveolar duct indicated by arrows) and well delineated interalveolar septae. (b) General aspect of interstitial pneumonitis with alveolar septae thickened by lymphomononuclear cells infiltration. (c) Free mononuclear cells (arrows), necrotic cell debris and proteinaceous exudate within the bronchioles lumen. (D) Presence of mononuclear cells lining the epithelium of a large bronchia. Note also the focal mild hyperplasia of the respiratory epithelium. HPS staining.

A
B
C
D

Figure 4.6

Histopathological findings in lungs of GST-ORF5 (a, b) and pRc-CMV5 (c, d) immunized pigs 14 days after challenge with PRRSV (IAF-Klop strain). (a) Localized region of intensive interstitial pneumonitis, and accumulation of macrophages and necrotic cell debris within the lumen of a bronchiole with apparently no apparent damages to the epithelium. (b) Normal aspect of the epithelium of a large bronchia with no accumulation of inflammatory exudate within the lumen. Note at the left, the presence of significant lesions of interstitial pneumonitis with alveolar septae thickened by lymphomononuclear cells infiltration. (c, d) Moderate interstitial pneumonitis with no apparent damages to the epithelium of the bronchioles and alveolar ducts (arrows). Absence of mononuclear cells and necrotic cell debris within the alveolar lumen. HPS staining.


A
ip
B
C
D

ARTTICLE 4

Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP_5 major envelope glycoprotein

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4.1 Summary

To determine the structural protein of the porcine reproductive and respiratory syndrome virus (PRRSV) involved in the production of neutralizing antibodies following clinical infection, correlation was studied between virus neutralization capability of convalescent pig sera and antibody response to the ORFs 3, 4, 5 and 7 encoded proteins GP_3, GP_4, GP_5 , and N, respectively. Individual virus genes were cloned into the pGEX-4T-1 vector and the recombinant viral proteins were expressed in *Escherichia coli* fused to the glutathione S-transferase protein. The resulting GST-ORF3, GST-ORF4, GST-ORF5 and GST-ORF7 recombinant fusion proteins were purified by electroelution and used as antigens for serological testing by indirect ELISA and Western Immunoblotting. The overall antibody (IgG and IgM) titers to PRRSV of pooled convalescent pig sera were first determined by indirect immunofluorescence (IIF), then sera with specific IgG titers > 1024 were tested for their specific virus neutralization activity and reactivity to individual recombinant fusion proteins. The data showed that, except for the early immune response (as revealed by the presence of specific IgM), neutralizing titers correlated with anti-GP_5 titers, but not with anti-GP_3 and anti-GP_4. The correlation between virus neutralization and anti-GP_5 titers was significant ($r=0.811$ and $p<=0.001$).

4.2 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important pig pathogen structurally and genomically related to the equine arteritis virus (EAV), the murine lactate dehydrogenase-elevating virus (LDV) and the simian hemorrhagic fever virus (SHFV) (Meulenberg *et al.*, 1993b; Plagemann and Moenning, 1992). These viruses have been recently classified in the genus *Arterivirus,* family *Arteriviridae* in the order *Nidovirales* which also comprises members of the family *Coronaviridae* (Cavanagh 1997). The genome of the PRRSV is a positive single-strand polyadenylated RNA molecule approximately 15 kb in length containing eight open reading frames (ORFs) which are transcribed in the infected cells as a nested set of sub-genomic mRNAs (Conzelmann *et al.*, 1993; Meulenberg *et al.*, 1993b). The enveloped viral particles contain three major structural proteins, a glycosylated envelope protein of 25 kDa (GP_5), an unglycosylated membrane (M) protein of 19 kDa, and a nucleocapsid (N) protein of 14 kDa, encoded by ORFs 5, 6 and 7, respectively (Mardassi *et al.*, 1995; Mardassi *et al.*, 1996; Meulenberg *et al.*, 1995). The ORFs 2 , 3 and 4 of the Lelystad virus, the European prototype of PRRSV, also encode for putative membrane-associated glycoproteins, GP_2, GP_3 and GP_4, with respective molecular masses of 30, 45, and 31 kDa (Meulenberg *et al.*, 1995).

Kinetics of development of antiviral and neutralizing antibodies to PRRSV following clinical infection in pigs have been previously studied (Loemba *et al.*, 1996; Nelson *et al.*, 1994). The determination of the protein(s) responsible for the induction of neutralizing antibodies is a key element to consider for the development of an efficient recombinant vaccine. Several reports clearly established that the ectodomain of the G_L glycoprotein of EAV, the counterpart of the GP_5 of PRRSV, contains immunodominant epitopes that trigger the immune system to produce neutralizing and protecting antibodies in horses (Balasuriya *et al.*, 1995a; Chirnside *et al.*, 1995a). Accordingly, monoclonal antibodies (MAbs)

directed against linear non-glycosylated antigenic determinants of the major GP_5 envelope glycoprotein of PRRSV can neutralize viral infectivity in vitro (Pirzadeh & Dea, 1997). Other investigators recently reported the production of neutralizing MAbs directed to the GP_4 of the Lelystad virus (Meulenberg *et al.*, 1997a; van Nieuwstadt et al., 1996). Recombinant baculoviruses expressing the ORFs 3 and 5 products of a Spanish strain apparently also conferred partial protection against reproductive problems in pregnant sows (Plana-Durán *et al.*, 1997b).

The purpose of the present study was to further investigate the viral protein(s) associated with virus neutralization in the course of a natural infection in pigs. A panel of pooled sera obtained from different pig farms in Quebec was screened by ELISA and Western Immunoblotting using recombinant *E. coli*-expressed glutathione S-transferase (GST) fusion proteins. A correlation between virus neutralization titers of convalescent sera and the presence of anti-GP_5 antibodies was demonstrated, but such correlation was not found in the cases of anti-GP_3, anti-GP_4 and anti-N specific antibodies.

4.3 Materials and methods

4.3.1 Virus and extraction of genomic RNA

The Quebec reference cytopathogenic strain IAF-Klop of PRRSV (Mardassi *et al.*, 1995) was plaque-purified twice and propagated in MARC-145 cells (Courtesy of Dr J. Kwang, Agriculture Research Service USDA, Clay Center, Nebr), a clone of MA-104 cells highly permissive to PRRSV (Kim *et al.*, 1996). The virus strain yielded titers of $10^{5.4}$-$10^{6.5}TCID_{50}$/mL after five to ten successive passages in MARC-145 cells. Genomic RNA was extracted from concentrated extracellular virions by the one-step guanidium isothiocyanate-acid phenol method (Chomczynsky and Sacchi 1987).

4.3.2 Cloning and sequencing of individual virus genes

Individual virus genes (ORFs 3 to 7) were amplified by RT-PCR, as previously described (Mardassi *et al.*, 1995). Sequences of the oligonucleotide primers used for RT-PCR and their location on the virus genome are described in Table 5.1. These primers, containing two restriction sites for *Eco*RI (sense primers) and *Bam*HI (antisense primers) at their 5'end for directional cloning, were designed according to the sequence of the IAF-Klop strain of PRRSV (EMBL/GeneBank accession number AF003344 for ORF3, AF003345 for ORF4, and U64928 for ORF5 to ORF7 genes) (Mardassi *et al.*, 1995). The PCR amplified products were purified using the QIAGEN DNA extraction kit (QIAGEN, Chatsworth, CA.) digested with *Eco*RI and *Bam*HI and finally ligated into similarly treated pUC-19 (Pharmacia Biotech Inc., Baie d'Urfé, Qc, Canada) or pZero plasmid (InVitrogen, San Diego, CA) according to the manufacturer's directions. Sequence analysis of these recombinant plasmids demonstrated that no alteration or mutation had occurred as a result of PCR amplification.

5.3.3 Procaryotic expression and purification of recombinant fusion proteins

ORFs 3 to 5 were choosen as potentially interesting genes for virus neutralization, while ORF7 was added as a control for the ELISA tests. Individual virus genes corresponding to ORFs 3, 4, 5 and 7 were cloned into the procaryotic expression vector pGEX-4T-1. To produce GST-ORF3, GST-ORF4, GST-ORF5 and GST-ORF7 recombinant fusion proteins, competent *E. coli* strain BL21 (DE3 strain, Novagen, Madison, WI) were transformed by the respective recombinant plasmid using standard methods (Sambrook *et al.*, 1989), and plated on 2YT agar medium containing 2% D-glucose and 100 µg/mL of carbenicillin (Sigma-Aldrich Canada Ltd, Oakville, Ont, Canada). To prepare stocks of recombinant fusion

proteins, overnight cultures of the transformed bacteria were diluted 1:100 in 2YT medium and grown at 37° C to optical density of 1.0 at 600 nm. Protein expression was then induced by addition of 0.1mM IPTG to the culture medium. After 4 hr incubation at 37° C under vigorous agitation (300 rpm), cells were pelleted by centrifugation and resuspended in 1:25 of the initial culture volume of ice-cold PBS containing 1mg/mL lysozyme and 1mM phenyl methyl sulfonyl fluoride (PMSF), Boehringer Mannheim, Laval, Qc, Canada). Following addition of Laemmli disruption buffer 4X and boiling for 5 min, samples of the disrupted cell suspensions were analysed by electrophoresis on SDS- 12% polyacrylamide slabs gels, as described previously (Mardassi *et al.,* 1995). The bands corresponding to the GST-ORF3, GST-ORF4 and GST-ORF7 fusion proteins were then electroeluted from the gel at 60 V for 6 hr in electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3), then for one hour in the same buffer without SDS, using a Bio-Rad Electro-Eluter (Bio-Rad Lab. Ltd, Mississauga, Ont., Canada). The production of GST-ORF5 recombinant fusion protein was made with the following modifications. After induction and incubation at 37°C, cells were pelleted and resuspended in 1:25 of the initial culture volume in ice-cold PBS containg PMSF, to which Triton X-100 was added at a final concentration of 1% after a 20 min incubation at 0° C. Cells were disrupted by sonication after another 10 min incubation on ice and the fusion protein entirely in the form of inclusion bodies was washed twice with 0.5 M glycine-NaOH buffer, pH 9.2. The final pellet was solubilized in the same buffer containing 8M urea. The solubilized GST-ORF5 recombinant fusion protein was refolded by chromatography on G25 Sepharose column (Bio-Rad) and subsequently purified by affinity chromatography on glutathione-Sepharose 4B (Pharmacia Biotech Inc.) and eluted with reduced glutathione (20 mM glutathione, 150 mM Tris-HCl pH 9.6). Protein concentrations in antigen preparations were determined by protein dying and spectrophotometry (Bradford 1976). We were unable to produce the ORF2-encoded recombinant protein in pGEX-*E. coli* expression system.

4.3.4. Indirect Enzyme-linked immunosorbent assays (ELISA)

Individual PRRSV recombinant fusion proteins were used as antigens. Flat-bottom polystyrene microtitration plates were used as solid-phase adsorbents (Flow Laboratories Inc., Mississauga, Ont., Canada). Optimum concentrations of recombinant fusion proteins were determined by checkerboard titration, using monospecific porcine or rabbit hyperimmune sera obtained from a previous study for ORF5 and 7 (Mardassi *et al.*, 1996) and corresponded to a range of 0,1 to 0,5 μg of protein per well. Recombinant fusion proteins were diluted to the appropriate concentration in 100 mM sodium carbonate-bicarbonate buffer, pH 9.6. A total reaction volume of 100 μL was added in all microtitration wells. After incubation for 18 hours at 4° C, antigen-coated plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and saturated for 1 hour at 25°C with PBS containing 3% skim milk powder (PBS-3). Convalescent pig sera diluted in PBS-3 were then incubated in the antigen-coated wells at 25° C for 2 hours. After washing with PBS-T, goat's anti-porcine IgG horseradish peroxydase (HRP)-conjugate (Sigma-Aldrich Canada) was added at a final dilution of 1:1000 in PBS-3 and the microtiters plates were further incubated for 1 hour at 37° C. Following another washing step, the enzyme substrate solution, containing 0.08% of 3',3',5',5' tetramethylbenzidine (Sigma-Aldrich Canada) in 120 mM citrate buffer and 0.005% hydrogen peroxide (pH 5.0) was added. The reaction was stopped after 10 min at room temperature by the addition of 50 µL of H_2SO_4 2N to each well and absorbance values were read at 450 nm with a spectrophotometer (Spectrophotometer, Milton-Roy, Fisher Scientific, Canada). Endpoint titers were determined from the point at which the antibody-binding curve crossed the average adsorbance attributed to non-specific binding of the conjugated antibody for each plate.

4.3.5 Western immunoblotting and other serological tests

Recombinant fusion proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes (0.45 μm-pore size; Schleicher & Schüell, Xymotech, Mont-Royal, Qc, Canada), as previously described (Loemba *et al.*, 1996). After saturation with Tris-buffered saline (20 mM Tris, pH 8.0, 150 mM NaCl) containing 3% skim milk (TBS-3), blots were cut into strips that were incubated for two hours at room temperature in the presence of 1:50 dilution of tested porcine sera in TBS-3. After five washes in TBS containing 0,05 % Tween-20 (TBS-T), the membranes were incubated for one hour at 37° C in the presence of 1:2000 dilution of an alkaline phosphatase-conjugated goat anti-porcine IgG (Sigma-Aldrich, Canada) in TBS-3. After three washes in TBS-T and two washes in TBS, the immune reaction was revealed by incubating the strips in the enzyme substrate solution containing 0,033% nitroblue tetrazolium and 0,017% 5-bromo-4-chloro-3-indolyl phosphate in the alkaline phosphatase buffer (100 mM Tris, pH 9,5, 100 mM NaCl, 5 mM $MgCl_2$). The reaction was stopped after 10 to 20 min at room temperature by washing the strips with distilled water.

The indirect immunofluorescence (IIF) test, using acetone fixed PRRSV-infected MARC-145 cells, as well as the virus neutralization (VN) test were performed, as previously described (Loemba *et al.*, 1996; Yoon *et al.*, 1994). The IIF antibody titers were expressed as the reciprocal of the highest dilution of tested pig sera giving specific cytoplasmic fluorescence. The VN titers were expressed as the reciprocal of the highest serum dilution that neutralized cytopathic changes induced in MARC-145 cell monolayers by a constant dose of 100 $TCID_{50}$ of virus.

4.4 Results

As shown in Figure 5.1, the oligonucleotide primers that were selected for RT-PCR permitted the amplification of genomic fragments having the sizes predicted

from the nucleotide sequence of the 3' end of the PRRSV genome (Table 5.1). These amplified genomic fragments, containing the first ATG codon of the respective structural genes, were cloned in the pGEX-4T-1 procaryotic expression vector. Following incubation in the presence of IPTG, transformed *E. coli* cells expressed recombinant fusion proteins, essentially in the form of inclusion bodies. The molecular masses of recombinant fusion proteins analysed by SDS-PAGE were estimated to be 52,3 kDa for GST-ORF3, 43,4 kDa for GST-ORF4, 45 kDa for GST-ORF5, and 39,6 kDa for GST-ORF7, in accordance with the values determined previously from the amino acid sequences of the putative structural proteins of the IAF-Klop strain of PRRSV (Mardassi *et al.*, 1996). Serological identification of the expressed recombinant fusion proteins was confirmed by Western immunoblotting using the homologous porcine anti-PRRSV serum obtained from experimentally-infected pigs (Fig. 5.2). None of the porcine sera tested (SPF pigs and PRRSV-infected pigs) showed reactivity towards the unfused GST (26 kD) protein (data not shown).

Serum samples obtained from Québec pig farms that have experienced outbreaks of reproductive and respiratory problems, were screened by IIF for the presence of anti-PRRSV specific IgG and IgM antibodies. Nineteen pooled sera showing high specific IgG titers (> 1024) to PRRSV were selected and further tested for the presence of PRRSV neutralizing antibodies and for their reactivity by ELISA and Western immunoblotting towards GST-ORF3, GST-ORF4, GST-ORF5 and GST-ORF7 recombinant fusion proteins (Table 5.2). None of the three negative pooled porcine sera tested (2609,2620 and 2621) showed reactivity to the recombinant fusion proteins. Alternatively, all of the nineteen PRRSV-positive sera tested had antibodies against the GP_5 and N proteins of PRRSV, whereas the presence of antibodies against the GP_3 and the GP_4 was not constant. A total of 52,6 % (10/19) and 47,4% (9/19) of the pooled sera tested failed to react by Western blotting to the GST-ORF3 and GST-ORF4 recombinant fusion proteins, respectively. Comparable reactivity profiles towards recombinant fusion proteins were obtained by ELISA. Nine of the pooled sera tested had specific anti-PRRSV IgM titers $\geq$1:16, as detected by IIF. The latters were thus considered to

correspond to cases of early PRRSV infections and were subsequently discarded from the correlation analysis. Previous studies have demonstrated that VN antibodies to PRRSV are usually not detected until the third or fourth week post-infection, whereas IgM antibodies usually decline rapidly after the third week post-infection. (Loemba *et al.*, 1996; Nelson *et al.*, 1994). To depict the correlation between anti-GP_5 and virus-neutralization (VN) titers, mean $\log_{10}$ of VN titers were plotted as a function of $\log_{10}$ anti-GP_5 titers in ELISA. As shown in Figure 5.3, an apparently meaningful correlation could be established. Regression analysis performed with the set of pooled pig sera, for which the IgM PRRSV-antibody titers in IIF was <16 (Table 5.2), provided the following result: R correlation coefficient of 0.811, while analysis of variance with VN as dependent variable gave a F ratio of 21.11 and a probability level of 0.001, values that show the high significance of the correlation between both sets of data. When similar analyses were carried out with the results obtained with GP_3 and GP_4, R correlation coefficients between ELISA and VN titers of 0.56 and 0.69 were obtained, with F ratios of only 5.3 and 9.5, and probability levels of 0.05 and 0.01. Interestingly, a significant correlation (R correlation of 0.79) was obtained between the strength of the N antibody response by ELISA and the presence of neutralizing activity of the pooled sera. However, in the latter case, analysis of variance with VN as dependent variable gave a F ratio of only 10.4 and a low probability of 0.01.

4.5 Discussion

The use of viral recombinant proteins expressed in *E. coli* has been previously used for differential diagnosis between vaccinated and infected animals or to design type-specific serological assays. (Crabb *et al.*, 1995; Laviada *et al.*, 1995). The pGEX-4T expression system has been used by several authors to develop ELISA tests for the detection of antibodies targeted against viral proteins carrying major antigenic determinants (Chirnside *et al.*, 1995b; Clavijo & Thorsen 1995; Crabb *et al.*, 1995; Rimstad *et al.*, 1994). In the present study, several porcine sera were screened for the presence of antibodies against ORFs 3, 4, 5 and 7 products of PRRSV. The purposes of the study were i) to determine the antigenicity of those proteins (especially for ORFs 3 and 4) , and ii) to confirm

that antibodies to the GP_5 are indeed responsible for virus neutralization in natural PRRSV infection.

Even if all pools of convalescent pig sera reacted by Western blotting towards the ORF 6-encoded viral matrix protein (data not shown), the latter was not included in this study since it has been previously demonstrated that it is not involved in virus neutralization (Dea *et al.*, 1996). The results obtained herein clearly indicated that all pools of sera tested reacted against the GP_5 and N proteins of PRRSV. However, not all sera reacted to the GP_3 and the GP_4 proteins. This result can be explained by a poor antigenicity, or by a difference in the kinetics of expression of those proteins during the viral cycle. It has been demonstrated that GP_3 and GP_4 are present only at low quantities on the viral particles (van Niewstadt *et al.*, 1996) and therefore it may be anticipated that antibodies to the latter two viral glycoproteins are not abundantly produced in the course of a clinical infection. However, the possibility that epitopes may not be correctly expressed in the procaryotic system, which does not allow glycosylation and may not respect the conformation of the native viral proteins, can not be ruled out.

The GP_5 was the first candidate for virus neutralization for two reasons. First, it has been already demonstrated by several investigators that antigenic determinants of the major envelope glycoprotein G_L of equine arteritis virus, the prototype virus of the *Arterivirus* genus, are involved in virus neutralization and protection (Balasuriya *et al.*, 1995a; Chirnside *et al.*, 1995 a and b). Second, we demonstrated previously that monoclonal antibodies to the GP_5 of PRRSV, the counterpart of the EAV G_L envelope glycoprotein have been found to neutralize viral infectivity in vitro (Pirzadeh & Dea 1997). Accordingly, the data obtained in the present study showed the existence of a significant correlation between the presence in pig sera of neutralizing antibodies to PRRSV and their reactivity towards the ORF 5-encoded protein. The correlation obtained was comparable to that previously reported in the case of EAV (Chirnside *et al.*, 1995b). However, the relatively high binding titers and low levels of neutralization suggest that most epitopes are not involved in neutralization. Moreover, it has been recently demonstrated that neutralizing MAbs raised against the recombinant GST-ORF5 fusion protein of the IAF-Klop strain of PRRSV show different reactivity profiles to North American field isolates (Pirzadeh *et al.*, 1997), and consequently testing the neutralizing activity of convalescent pig sera toward the GP_5 of an heterologous strain may result in lower titers.

On the other hand, R correlation coefficient of lower significance were obtained between VN antibody titers and ELISA titers to either the GP_3 or the GP_4. These results are controversial with previous findings on the existence of a neutralization domain in the GP_4 of LV which however was found to induce only low level of antibodies in natural infection. (Meulenberg *et al.*, 1997a; van Niewstadt *et al.*, 1996). It can thus be speculated that while the GP_4 may bear neutralizing epitopes, the major neutralizing domains of PRRSV are associated with the GP_5. Since a low level of amino acid sequence identity exists between the structural proteins of European and North American isolates of PRRSV, (Mardassi *et al.*, 1995; Murtaugh *et al.*, 1995), it remains to be demonstrated if such antigenic determinants associated to virus neutralization also exist on the ORF 4 product of North American strains. The level of amino acid identity of the GP_4 of strains from both continents is only of 64 percent (Kwang *et al.*, 1994). Therefore the role of the GP_4, as well as that of the GP_3, of the North American isolates in virus neutralization and protection against PRRSV infection still remains to be established.

The high R correlation coefficients determined between ELISA antibody titers to the N protein and the presence of neutralizing activity of the pooled sera is questionable, since it has been previously demonstrated that monoclonal antibodies directed towards epitopes of the N protein are non-neutralizing antibodies (Benfield *et al.*, 1992b, Dea *et al.*, 1996). However, N protein is the more abundant structural protein of the virion and it has been demonstrated to be highly immunogenic (Molitor *et al.*, 1997; Nelson *et al.*, 1994). Serum antibodies against the N protein appear as early as 11 days post-infection and thereafter are detectable for approximately six months, as shown by IIF, Western blotting and ELISA (Joo *et al.*, 1997; Motitor *et al.*, 1993; Yoon *et al.*, 1995). Study of the kinetics of the humoral immune response to PRRSV have already shown that neutralizing antibodies are usually not detected until the third or fourth weeks post-infection, (Loemba *et al.*, 1996; Yoon *et al.*, 1994), when infected pigs have already developed maximal IIF antibody titers. This would explain why a correlation was obtained herein between the strength of the N antibody response, as determined by ELISA and Western blotting and the presence of neutralizing activity of the pooled serums.

Table 5.1 Sequence of the oligonucleotide primers used for RT-PCR and size of the expected amplified products.

Primer designation	Sense	Sequence 5' to 3'	Genome Location* (Bases)	Product size (bp)	Gene size† (bp)
ORF2-S	+	ATGAAATGGGGTCTATGC	28 - 45		
ORF2-AS	-	CACACCGTGTAATTCACCG	793 - 811	783	768
ORF2-S	+	ATGGCTAATAGCCGTACA	651 - 668		
ORF3-AS	-	CTATCGCCGTGCGGCACT	1398 - 1415	765	762
ORF4-S	+	ATGGCTGCGTCCCTTCTT	1196 - 1213		
ORF4-AS	-	TCAAATTGCCAACAGAATGG	1713 - 1732	537	534
ORF5-S	+	ATGTTGGGGAAATGCTTGACC	1743 - 1763		
ORF5-AS	-	GGCAAAAGTCATCTAGGG	2339 - 2355	612	600
ORF6-S	+	ATGGTGTCGTCCTAGATGAC	2337 - 2357		
ORF6-AS	-	CAGCTGATTGCATGGCTGGC	2897 - 2916	579	522
ORF7-S	+	CTAAATATGCCAAATAACAAC	2845 - 2865		
ORF7-AS	-	CTCAAGAATGCCAGCTCA	3223 - 3240	396	369

* Nucleotide location on the PRRSV genome was given in reference to the sequences of the prototype North American strains ATCC VR-2385 (EMBL/GenBank accession numbers U20788 and U03040) and ATCC VR-2332 (EMBL/GenBank accession number U00153) (Meng *et al.*, 1994 and 1995a; Murtaugh *et al.*, 1995).

† Sizes of individual viral genes refer to those deduced from the nucleotide sequences of the tissue culture-adapted Quebec IAF-Klop strain of PRRSV (EMBL/GenBank accession numbers are given in the text Mardassi *et al.*, 1995).

Table 5.2 Comparison of the reactivity of control and convalescent pig sera from 19 pig herds in Quebec to PRRSV-infected cells by IIF and SN and to recombinant fusion proteins by Western immunoblotting and ELISA.

* Reciprocal of the highest dilution of pig serum at which specific cytoplasmic fluorescent was observed on PRRSV-infected MARC-145 cells.

† Reciprocal of the highest dilution of pig serum neutralizing cytopathic changes in MARC-145 cells by 100 $TCID_{50}$ of PRRSV, IAF-Klop strain.

‡Western immunoblotting: relative intensity of colorimetric reaction obtained from a 1:50 dilution of pig serum when tested to individual recombinant fusion protein adjusted to 0.1 μg per well.

¶ Log_{10} ELISA titers: endpoint titers of tested pig sera were defined as described in Materials and Methods

Serums	IIF titers*		VN†	Reactivity to fusion proteins (GST-ORF) by: Western blotting‡				log_{10} ELISA titers¶			
(pools)	IgG	IgM	titers	3	4	5	7	3	4	5	7
710	>1024	<16	<8	-	-	++	+	2.0	<1.7	2.0	1.7
794	>1024	64-256	<8	-	-	++	+	2.3	1.7	2.9	2.6
878	>1024	<16	<8	-	-	++	+	<1.7	<1.7	2.3	1.7
881	>1024	<16	<8	+	-	+	+	2.3	1.7	2.3	2.0
890	>1024	<16	8	-	-	+++	+	2.6	1.7	2.6	1.7
891	>1024	256	<8	-	+	+	+	2.0	2.3	2.9	2.6
893	>1024	16	64	++	+	+	+	2.9	2.0	2.9	2.6
904	>1024	<16	256	++	+	+++	++	3.2	2.6	3.8	3.5
908	>1024	<16	16	++	-	+	+	2.9	2.3	2.9	2.6
914	>1024	<16	64	+	+	+	+	2.6	2.3	2.6	2.6
916	>1024	16	<8	-	-	+	+	2.0	<1.7	2.3	2.0
918	>1024	<16	<8	-	-	+	+	2.3	1.7	2.3	2.3
919	>1024	<16	16	+	+	+	+	2.6	2.3	2.6	2.6
921	>1024	64	<8	+	+	+	+	1.7	2.0	2.0	2.0
922	>1024	64	64	+	+	++	+	2.3	2.3	2.6	2.6
929	>1024	64	<8	-	-	+	+	<1.7	<1.7	2.0	2.0
930	>1024	<16	8-16	+	+	+	+	2	2.3	2.3	2.0
931	>1024	64	<8	-	+	+	+	2.3	2.3	2.3	2.3
932	>1024	64-256	<8	-	+	+	+	2.0	2.3	2.3	2.6
2609	<16	<16	<8	-	-	-	-	<1.7	<1.7	<1.7	<1.7
2620	<16	<16	<8	-	-	-	-	<1.7	<1.7	<1.7	<1.7
2621	<16	<16	<8	-	-	-	-	<1.7	<1.7	<1.7	<1.7

Figure 5.1

Electrophoretic profiles in agarose gel of RT-PCR amplified products corresponding to the ORFs 2 to 7 genes of the IAF-Klop strain of PRRSV. The corresponding products are in lanes 2 to 7, with the molecular weight markers in lane 1. The sizes of genomic fragments amplified by RT-PCR are in accordance with those predicted from the nucleotide sequence of the reference IAF-Klop strain of PRRSV (Mardassi *et al.*, 1995)

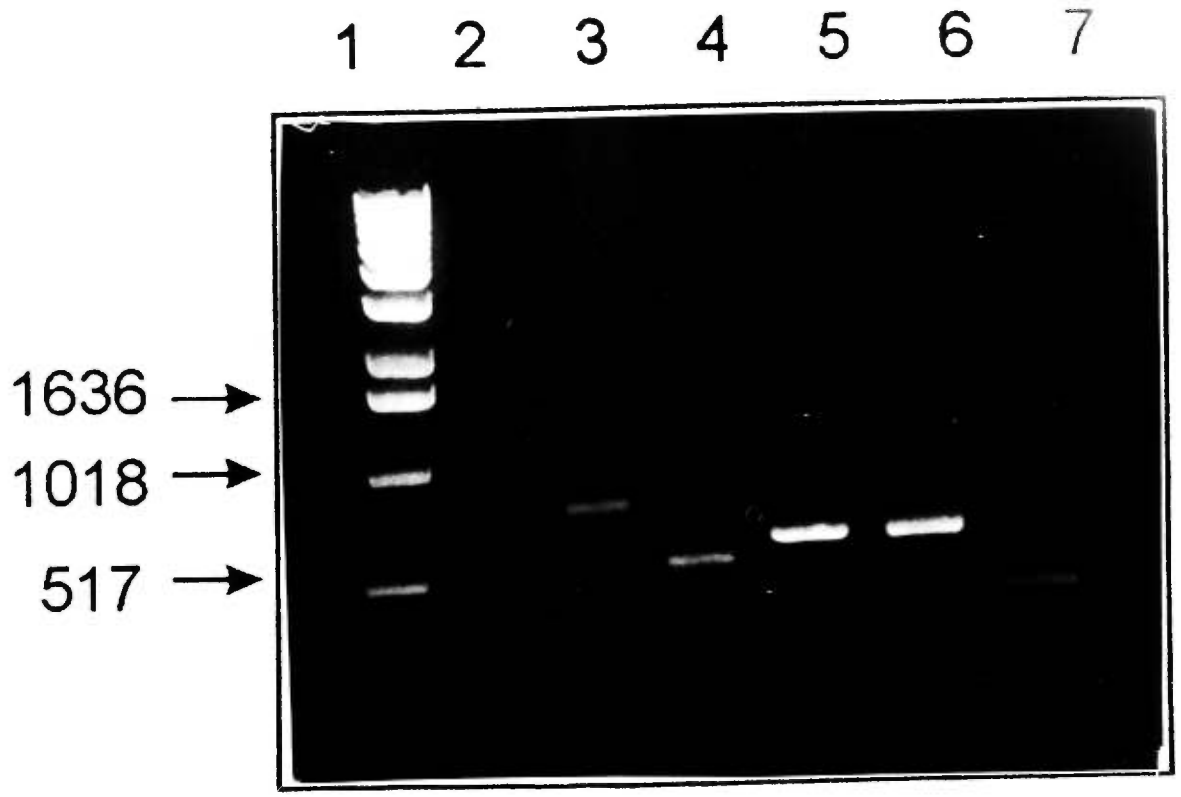
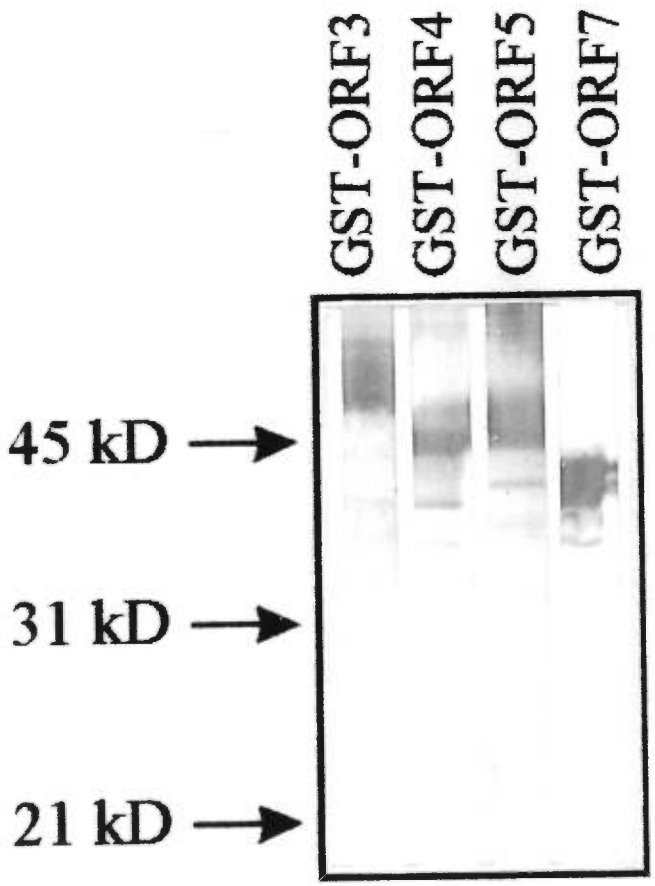

1
2
3
4
5
6
7
1636
1018
517

Figure 5.2

Western blotting analysis of the *E. coli* - expressed recombinant fusion proteins. The ORF3, 4, 5 and 7 of the IAF-Klop strain of PRRSV were cloned in the procaryotic expression vector pGEX-4-T-1 and the recombinant fusion proteins were synthesized in transformed bacteria in the form of inclusion bodies. The recombinant fusion proteins GST-ORF3, GST-ORF4, GST-ORF5 and GST-ORF7 were separated by electrophoresis on a 12% SDS-polyacrylamide gel and recovered by electroelution. Recombinant fusion proteins were electrophoretically transferred onto nitrocellulose membranes and immune complexes were revealed following incubation in the presence of the autologous porcine anti-PRRSV hyperimmune serum. The positions of the molecular weight markers are indicated on the left.


GST-ORF3
GST-ORF4
GST-ORF5
GST-ORF7
45 kD
31 kD
21 kD

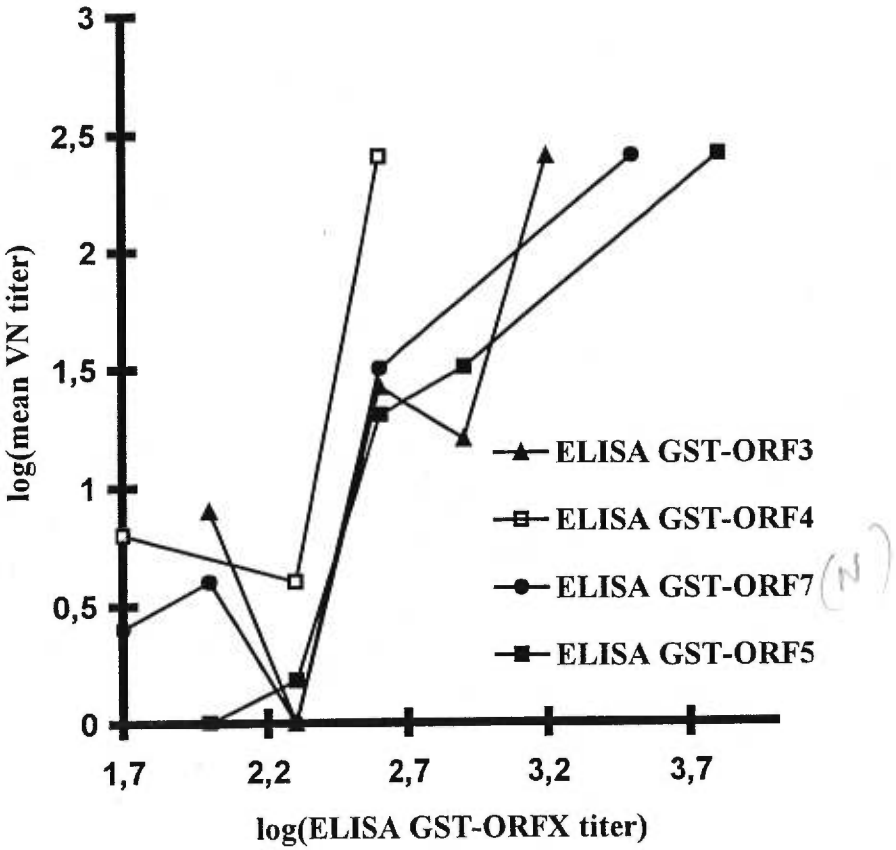


Figure 5.3

Correlation analysis between anti-GP_3, GP_4, GP_5 and N serum titers in ELISA and neutralizing convalescent PRRS porcine sera.

CHAPTER 4

EPITOPE MAPPING OF THE MAJOR ENVELOPE GLYCOPROTEIN (GP_5) OF PRRS VIRUS BY GENERATING TRUNCATED PROTEINS

Development of truncated ORF5 genomic library of PRRSV and the expression of the truncated genes in prokaryotic expression system.

6.1 Introduction

The objective of this part of our research work was to develop a series of truncated ORF5 genes of PRRSV for expression in prokaryotic system *E. coli*. These truncated proteins were later to be used for epitope mapping of the GP_5 protein of PRRSV by establishing the reactivity of these proteins with MAbs reported in article one of this thesis (Pirzadeh & Dea 1997) and porcine neutralizing convalescent sera by WB and ELISA. It was realized that the ORF5 encoded protein expressed in *E. coli* may not be conform with the native GP_5 of PRRSV due to post-translation modifications and the addition of oligosaccharides residues to the latter. However, the reactivity of porcine convalescent sera and anti-ORF5 MAbs with the *E. coli* expressed recombinant protein in WB and ELISA (Gonin *et al.*, 1997; Pirzadeh & Dea 1997a) justified our strategy. According to our previous results (Pirzadeh and Dea 1997a), some of the neutralizing epitopes associated with the GP_5 are continuous and they are not affected by absence of oligosaccharides residues on the GP_5. Our truncation strategy consisted of amplifying the designated areas of ORF5 by internal oligonucleotide primers complementary to specific sequences located at 5' and 3' extremities of these areas. A Kozak's motive was added to forward primers in anticipation of future expression in eukaryotic systems and *Bam*HI restriction sites were added to both forward and reverse primers for cloning into compatible sites of prokaryotic expression vectors. The part excised from 5' extremity of the ORF5 coding region consisted of 25 aa of the signal sequence (Pirzadeh *et al.*, 1997). Excised segments from 3' end of ORF5 consisted of fragments of 40-50 aa in length. The truncation points were selected in regions 5' to amphipathic areas of ORF5 which are predicted to be associated with α helix formation and thus favoring formation of linear or continuous epitopes (Cornette *et al.*, 1987;

Margalit *et al.*, 1987). The reactivity pattern of limited number of PRRSV infected convalescent neutralizing sera indicate that the amphipathic region located at 3' extremity of ORF5 is associated with neutralizing domains. Personally, I could not pursue this part of our investigations since the murine ascites corresponding to MAbs IAF-8A8, IAF-1B8, IAF-2A5, IAF-3A12 and IAF-1C10 reported in our previous paper (Pirzadeh and Dea 1997a) were available in limited quantities, just enough for manipulations necessary to complete the first two articles reported here. Only the first two MAbs (IAF-8A8, IAF-1B8) but not the last 3 MAbs (IAF-2A5, IAF-3A12 and IAF-1C10) could be successfully suncloned, and they were inoculated to mice for production of ascitic fluides. For inexplicable reasons, the new batches of ascites originating from the new subclones of MAbs IAF-8A8 and IAF-1B8 showed only weak reactivity in ELISA and WB with the *E. coli* expressed ORF5. These two MAbs, reacted specificly with MARC-145 PRRSV-infected cells, with titers ranging from 25,600 to 51,200 and neutralized viral PRRSV infectivity *in-vitro* with titers ranging 32-64. Therefore, the pursuit of this part of the project was interrupted and it's resumption will be pending on development of new MAbs.

6.2 Materials and Methods

PCR amplification, cloning and plasmid screening were performed according to standard procedure (Sambrook *et al.*, 1989). Induction of transformed *E. coli* for expression of the fusion proteins has been performed according to procedure already described (Pirzadeh et Dea 1997, Pirzadeh *et al.*, 1997).

5.2.1 Oligonucleotide primers

The following oligonucleotide primers were used for amplification of selected areas of ORF5:

Designation	Sequence
ETS5.2 *(1-21)→	5'-*GGA,TCC,* **GCC,GCC,GCC,**ATG,TTG,GGG,TGC ,TTG,ACC-3'
5TRS1 *(79-86)→	5'-*GGA,TCC,***GCC,GCC,GCC,**ATG,GCG,CTC,GTC,AAC-3'
5TRR1 *(474-457)←	5'-*GGA,TCC,*CTA,GGG,TGA,CCG,CCA,ACG,ATA-3'
5TRR2 *(342-325)←	5'-*GGA,TCC,*CTA,GAC,AGC,GTA,GAC,GCT,ACT-3'
5TRR3 *(246-229)←	5'-*GGA,TCC,* CTA,GAG,GGC,GCC,ATA,GGA,GAC-3'
5GSI1 *(612-598)←	5'-*GGA,TCC,*GGC,AAA,AGT,CAT, CTA,GGG-3'

* Figures in brackets indicate the position of oligonucleotides on the ORF5 coding area. Arrows indicate the orientation of primers. Italic characters indicate *Bam*HI restriction sites in the primers, bold characters feature Kozak's motif (Kozak 1987), single underlined sequence are borrowed from positive or negative strand of ORF5 cDNA for hybridization of primers, double underlined codons feature sequence situated in the ORF6 coding region of PRRSV and shaded sequences indicate stop codons. By various combinations of the above primers, we generated five different truncated genes designated Tr1 to Tr5 as described in Table 6.1 and schematized in Figure 6.1. The hydropathy profile of the expected ORF5 truncated recombinant proteins are depicted in Figure 6.2.

5.2.2 Expression vectors and *E. coli* strain

PCR-amplified fragments and plasmidic vectors were digested by *Bam*HI and ligated according to normal procedure. DH5α strain of *E. coli* were used to amplify and screen the recombinant plasmids. Two expression vectors, pGEX-4T1 (Pharmacia Biotech) and pET-21a (Novagene) were used for production of

recombinant proteins in DE3-BL21 strain of *E. coli*. pGEX expression vector encodes a fusion protein consisting of GST with an apparent M_r of 26 kDa joined by its C-terminal to the target protein. Cells transformed by the recombinant pET-21a express the target protein fused to six histidine residues by it's 3' extremity. The apparent M_r of the fusion protein expressed in this system was approximately one kDaa greater than the target protein. Initially, all five truncated genes were expressed in pGEX but the level of expression was insignificant for the proteins designated as Tr1, Tr2 and Tr3. Furthermore the protein designated as Tr3 and fused to GST, had an apparent M_r of 35-36 kDa and intermingled a prominent bacterial protein band. These inconveniences of pGEX expression system prompted us to use pET-21a as an alternative vector for expression of the first three truncated genes (Tr1, Tr2 and Tr3).

5.2.3 Purification of fusion proteins and WB

Irrespective of the expression system employed, the recombinant proteins were synthesized entirely and accumulated within the *E. coli* cells in form of inclusion bodies. Following induction by 0.5 mM of IPTG at optical density of 0.8-1.0 at 600 nm, cultures of DE3-BL21 *E. coli* cells were incubated at 37° C for 5 hours with vigorous shaking, and then were pelleted and resuspended in 1/25 original culture volume of PBS containing 0.1 mM PMSF. After disrupting cell walls by sonication, inclusion bodies were precipitated by centrifugation at 10,000 x g for 15 min and washed twice with 0.5 M glycine NaOH buffer, pH 9.2, containing 0.1 mM PMSF. The final pellets were resuspended in 5X Laemmli's sample buffer (1 mL of sample buffer for inclusion bodies from 50 mL of culture of pET-21a transformed bacteria or 25 mL of pGEX-transformed bacteria), boiled for five minutes and separated by SDS-PAGE (1.5mm thick, 75μL per well). The recombinant proteins were subsequently extracted from the gels by electroelution, using a Bio-Rad model 422 Electro-Eluter. An optimal polyacrylamide

concentration of 12% was used for all fusion proteins with the exception of Tr3-pH for which a 15% gel produced a better separation.

Electro-elutions were performed overnight at 4°C with running buffer containing PMSF (0.1 mM final concentration) and where applicable, the purified proteins where reacted with rabbit anti-GST hyperimmune sera for establishing the purity and specificity of the protein. SDS-PAGE analysis of the proteins confirmed that the purified recombinant fusion proteins were not contaminated by *E. coli* proteins and their estimated M_r corresponded to that expected from sequencing data.

WB was performed as described previously (Pirzadeh & Dea 1997) but the following modification procedure was made to avoid confusion resulting from the reactivity of porcine sera or murine ascitic fluids with *E. coli* contaminant proteins that co-purified with the recombinant proteins. Sonicates of untransformed DE3-BL21 *E. coli* were produced according to the same procedure mentioned for transformed bacteria. The cell lysates (750 μL) was then mixed with an equal volume of 1/25 dilution of test sera or ascites and 50 μL Protein A-Sepharose CL4B was added to the mixture. Following an overnight incubation at 4° C with moderate agitation, the mixtures were centrifuged at 10,000xg for 10 min and the supernatant were used as 1/50 dilution of serum for WB.

Table 6.1: Number of aa and the calculated M_r of truncated ORF5 proteins.

Protein segment and designation	Primers combination		Number of Amino Acids	Predicted M_r, kDa
	Forward	Revers		
ORF5 Entire	ETS5.2	GSI1	200	22.4
Tr1-pH*	ETS5.2	5TRR1	175	17.3
Tr2-pH*	5TRS1	GSI1	158	19.2
†GST-Tr3, Tr3-pH*	5TRS1	5TRR1	133	14.6
†GST-Tr4	5TRS1	5TRR2	89	9.4
†GST-Tr5	5TRS1	5TRR3	57	5.0

* Expressed in pET-21a vector and fused to six histidine residues, increasing the apparent M_r by 1 kDa.

† Expressed in pGEX-4T vector and fused to GST. The apparent M_r of the fusion protein on SDS-PAGE is 26 kDa higher than the calculated M_r indicated on the right column.

5.3 Results

Figures 6.3, 6.4 and 6.5 illustrate the SDS-PAGE migration profiles of *E. coli* expressed ORF5 truncated proteins, using pGEX-4T or pET-21a procaryotic expression vectors.

As it was previously indicated, after subcloning by limiting dilution method and production in murine ascitic fluid, none of the two MAbs IAF-1B8 and IAF-8A8 reacted with the *E. coli*-expressed recombinant GST-ORF5 or ORF5-pH in WB or ELISA (data not shown). However, several pools of porcine convalescent neutralizing anti-PRRSV sera were available from previous experiences (Gonin *et al.*, 1998) reacted in WB with the entire recombinant ORF5 or the protein

designated Tr2 (Figures 6.6). The Tr2, corresponds to the entire ORF5 encoded protein, from which the 5' signal sequence was deleted. The reactivity of the porcine sera with the ORF5 recombinant fusion proteins was lost as a consequence of the deletion of 42 aa located at the 3' extremity of the ORF5- encoded protein. We would therefore anticipate that at least one antigenic domain consisting of linear continuous epitope(s) be located at this part of ORF5. This speculation is supported by our preliminary results in WB with the truncated proteins and the amphipathicity profile of the carboxy-terminal of the ORF5 which is formed by small regions of alternating hydrophobic and hydrophilic peptides that favours α helix and subsequently immuno-dominant epitope formation. However, these epitopes may not necessarily be implicated in virus neutralization. Conformational discontinous epitopes which are undetectable by WB and located elsewhere on GP_5 may also be implicated in virus neutralization. This view is supported by our previous observations that following exposure to PRRSV, GP_5 specific antibodies are detectable by WB in porcine sera as early as PID seven whereas neutralizing antibodies appear three to four weeks after experimental exposure to PRRSV. Further investigations with a large panel of anti-ORF5 MAbs and a greater number of porcine convalescent anti-PRRSV neutralizing sera are required to confirm these preliminary results.

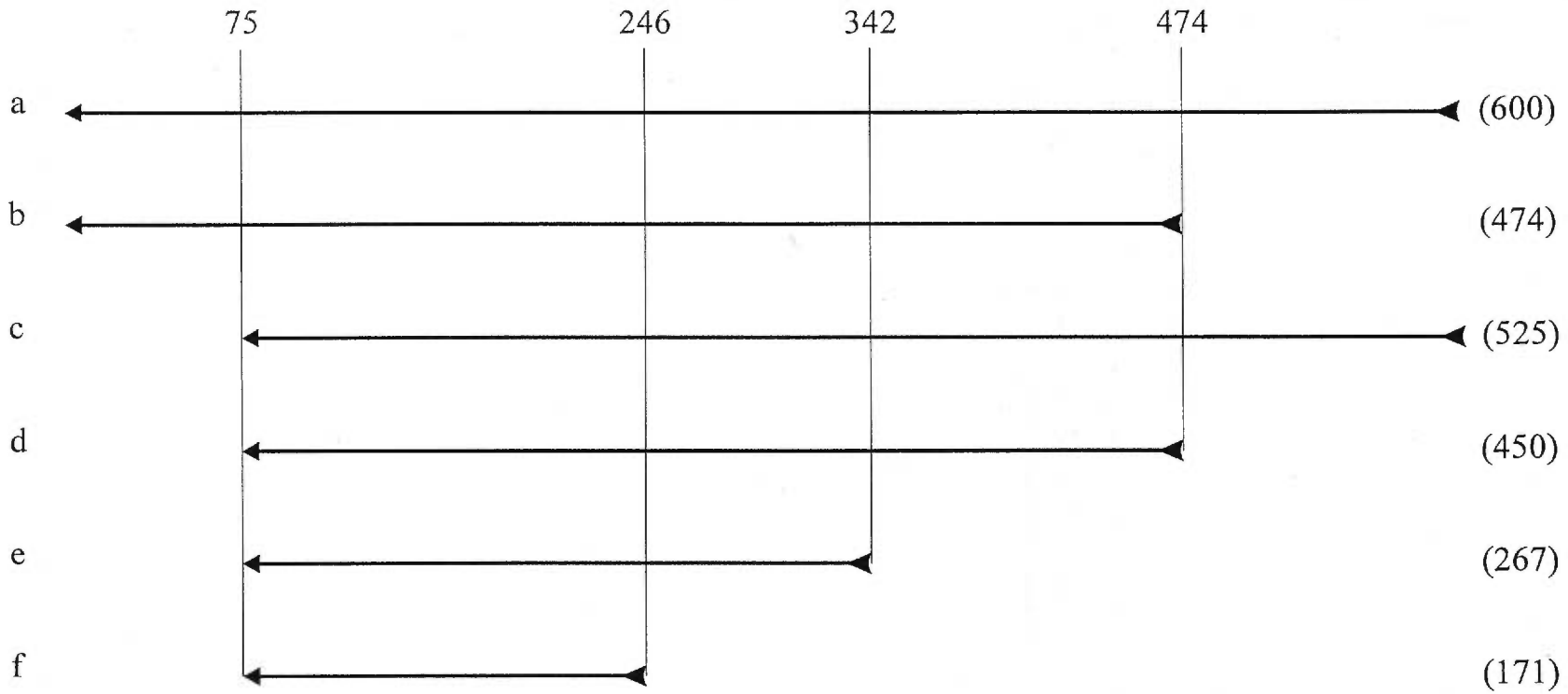


Figure 6.1
ORF5 entire encoding area (a) and truncated genes 1 to 5 (b – f). Truncation sites are indicated on (a). Figures in parentheses indicate the length of the truncated genes in nt.

Figure 6.2

Hydropathy profiles of the ORF5 truncated translation products of IAF-Klop, compared to the entire ORF5 coding region. Profiles were determined by the method of Kyte and Doolittle (1982) for the average hydropathy of overlappinf segments of 11 aa. The horizontal line separates the hydrophobic regions (above the line) from the hydrophilic regions 'below the line).

a: Entire ORF5 coding region.

b: Tr1, aa 1-158.

c: Tr2, aa 26-200.

d: Tr3, aa 26-158.

e: Tr4, aa 26-114,

f: Tr5, aa 26-82

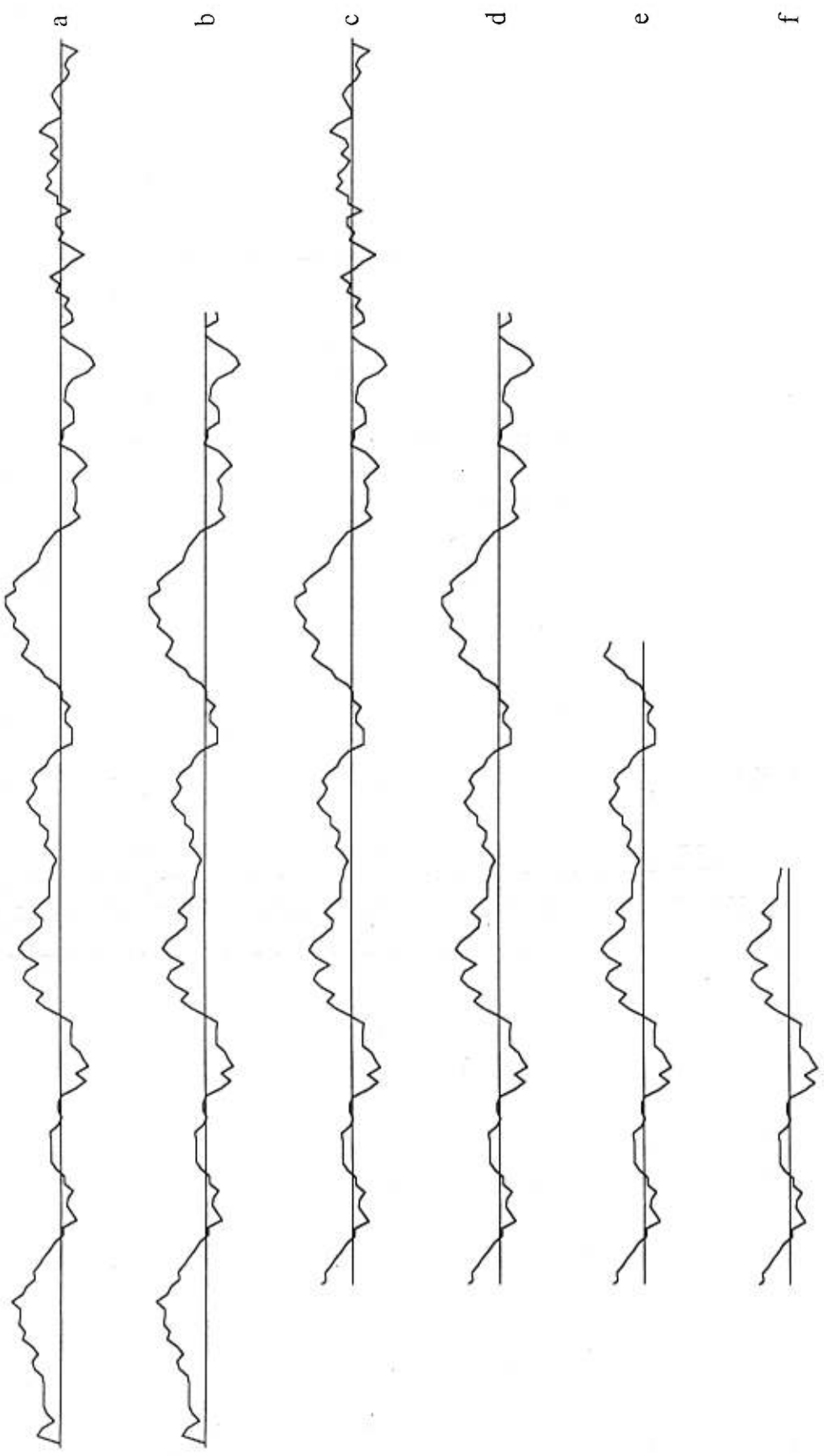

a
b
c
d
e
f

Figure 6.3

SDS-PAGE analysis of truncated GST-ORF5 recombinant fusion proteins expressed in *E. coli*. Truncated ORF5 genes were cloned in procaryotic expression vector pGEX-4T1, DE3-BL21 cells were transformed by the recombinant plasmid. When the target gene is cloned into the plasmid in the correct orientation, transformed cells express a fusion protein consisting of the target protein joined by its N-terminal to GST. Otherwise, only GST is expressed.

(A) Lanes 1-10 and 12: expression of GST alone. Lane 11: expression of GST-TR3 fusion protein. Lane 13: untransformed BL21 cells.

(B) Lanes 2,3,5,6,8,10,11 and 12: expression of GST alone. Lanes 4,7 and 9: expression of GST-TR4 fusion protein. Lanes 1 and 13: untransformed BL21.

(C) Lanes 3,5,6,9 and 10: expression of GST alone. Lanes 1,2,4,7,8 and 11: expression of GST-TR5. Lane 12: untransformed BL21.

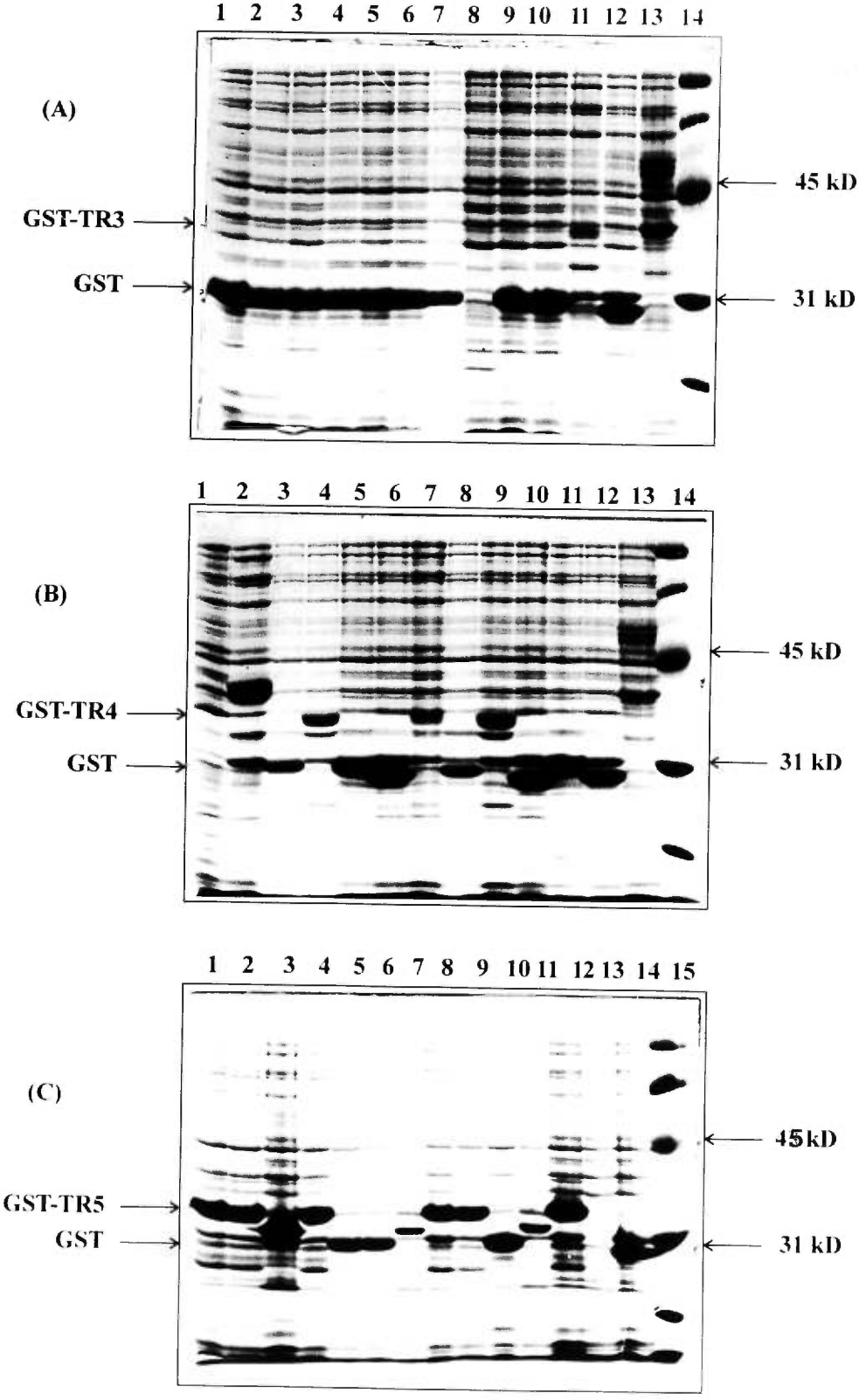

1 2 3 4 5 6 7 8 9 10 11 12 13 14
(A)
GST-TR3
GST
45 kD
31 kD
1 2 3 4 5 6 7 8 9 10 11 12 13 14
(B)
GST-TR4
GST
45 kD
31 kD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
(C)
GST-TR5
GST
45kD
31 kD

Figure 6.4

SDS-PAGE analysis of truncated ORF5-pH recombinant fusion proteins expressed in *E. coli* transformed by pET21a expression vector.

Lanes 1 and 2: expression of Tr2-pH. Lanes 3-6: expression of Tr1-pH protein.

Lane 7: untransformed BL21.

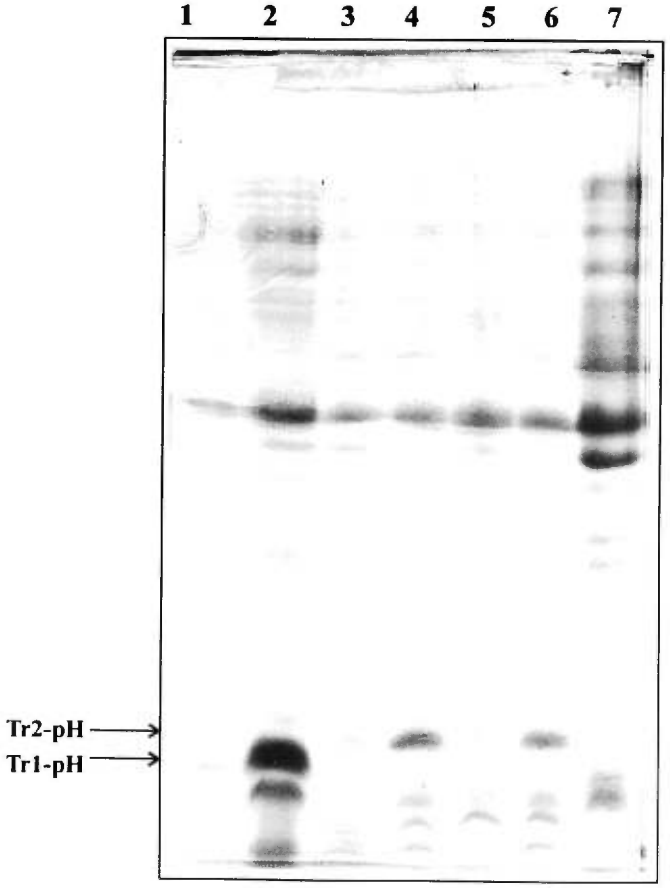

1
2
3
4
5
6
7
Tr2-pH
Tr1-pH

Figure 6.5

SDS-PAGE analysis of truncated GST-ORF5 recombinant fusion proteins expressed in *E. coli* transformed by pGEX expression vector.

(A) Lane 1: Untransformed BL21. Lane 2:expression of GST-TR3. Lane 3: expression of GST-TR4. Lane 4: expression of GST-TR5

(B) WB reactivity pattern of anti-GST rabbit sera with fusion proteins expressed in BL21 cells transformed by pGEX4T1 recombinant expression vectors. Lane 1: GST-ORF5. Lane 2: GST-TR5. Lane 3: GST-TR4. Lane 4: GST-TR3.

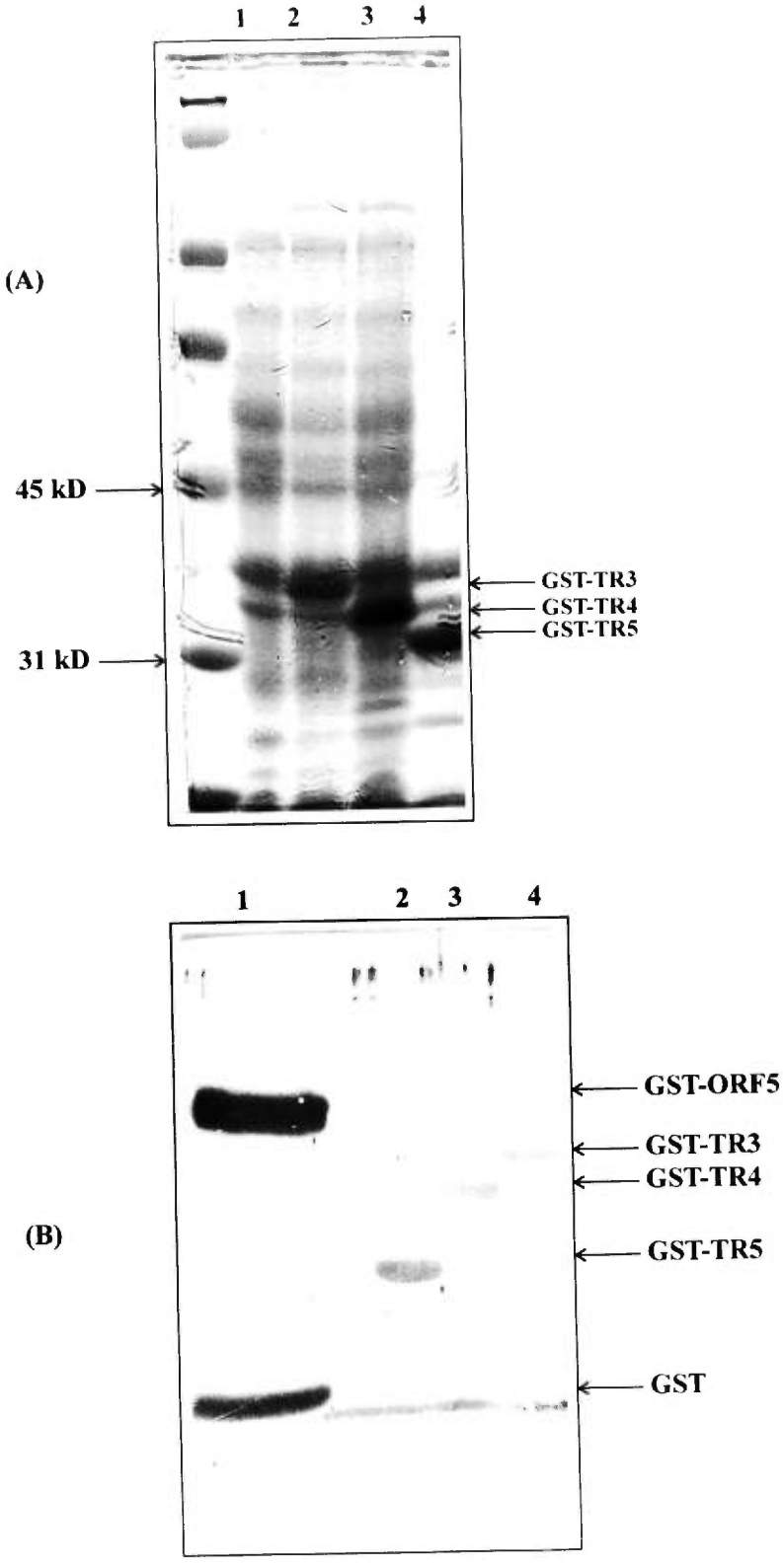

1
2
3
4
(A)
45 kD
31 kD
GST-TR3
GST-TR4
GST-TR5
1
2
3
4
(B)
GST-ORF5
GST-TR3
GST-TR4
GST-TR5
GST

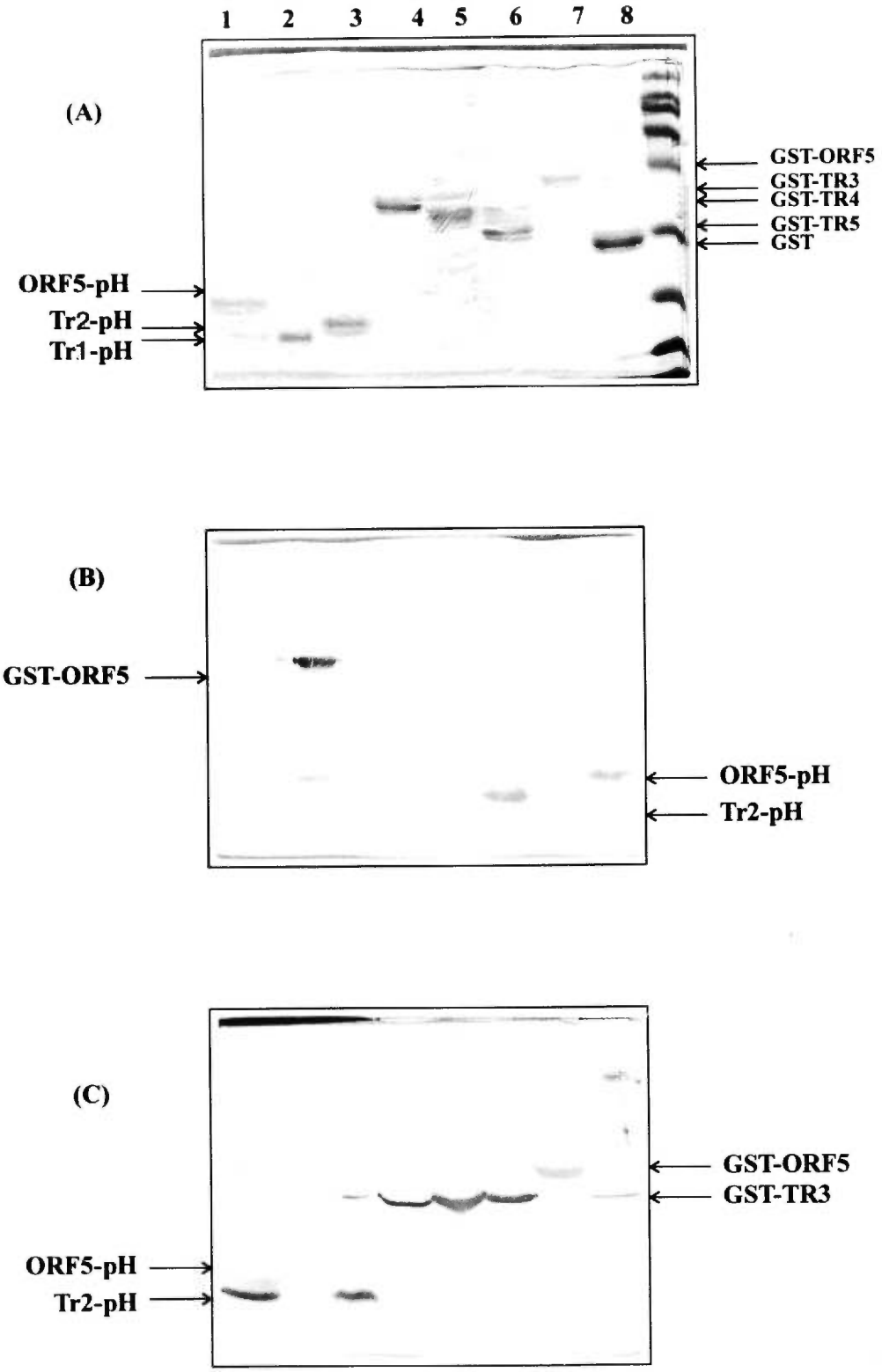
Figure 6.6

SDS-PAGE analysis of purified truncated fusion proteins and WB reactivity pattern of neutralizing convalescent anti-PRRSV positive pig sera.

(A) Lane 1: ORF5-pH. Lane 2: Tr1-pH. Lane 3: Tr2-pH, all expressed in BL21 transformed by recombinant pET21a expression vector.
Lane 4: GST-TR3. Lane 5: GST-TR4. Lane 6: GST-TR5. Lane 7: GST-ORF5. Lane 8: GST.

(B) Duplicate of polyacrylamide gel shown on A and transferred to nitrocellulose membrane for WB reactivity with a pool of pig convalescent sera positive for anti-PRRSV neutralizing antibodies. Remark that the serum reacts only with the entire ORF5 encoded recombinant protein or Tr2 with intact C-terminal. No other fusion proteins shown on (A) had any reactivity with the serum as a result of excision of its C-terminal.

(C) Reactivity patterns of the same pool sera before adsorption on *E. coli* proteins with the duplicate of membrane shown on (B). Remark the nonspecific reactivity as a result of bacterial protein contaminants that comigrated with the purified GST-ORF5 recombinant fusion proteins.


1 2 3 4 5 6 7 8
(A)
GST-ORF5
GST-TR3
GST-TR4
GST-TR5
GST
ORF5-pH
Tr2-pH
Tr1-pH
(B)
GST-ORF5
ORF5-pH
Tr2-pH
(C)
GST-ORF5
GST-TR3
ORF5-pH
Tr2-pH

PART III

GENERAL DISCUSSION

AND CONCLUSION

Since early isolation of PRRSV strains in Europe and North America, the presence of three major structural proteins with an apparent molecular masses of 25, 19 and 15 kDa in cell lysates of PRRSV-infected cells were reported and designated as the major structural proteins of PRRSV (Mardassi *et al.*, 1994a; Meulenberg *et al.*, 1993b; Nelson *et al.*, 1993). Later, it was established that the above mentioned proteins are encoded by ORFs 5 to 7, respectively. Recent findings on structural proteins of LV indicate that the translation products of ORFs 2 to 4 are also incorporated into the viral particles. Most PRRSV-infected convalescent sera strongly react with the three major structural proteins (Loemba *et al.*, 1996). Some sera have also been shown to react with the GP_3 and GP_4 of PRRSV (Gonin *et al.*, 1998, Kwang *et al.*, 1994). However the role of these structural proteins in inducing neutralizing antibodies in naturally-infected or vaccinated animals and conferring protection against reinfection with PRRSV, is not yet fully understood.

Unlike PRRSV, the role of ORF5-encoded envelope glycoprotein of other Arteriviruses in triggering production of neutralizing antibodies in their natural hosts were already recognized. For example, the ectodomain of the G_L glycoprotein of EAV, which is located at its N-terminal half, contains a highly immunogenic region consisting of not more than 44 amino acid residues (Chirnside *et al.*, 1995a). An immunodominant epitope is mapped to this region which induces neutralizing antibodies in horses (Balasuriya *et al.*, 1995a, Chinside *et al.*, 1995a, St-Laurent *et al.*, 1997). Immunizing horses with an *E. coli*-expressed recombinant protein encoded by this region of G_L or a synthetic peptide representing close to 50% of this immunogenic region induces EAV-neutralizing antibodies in vaccinated horses (Chirnside *et al.*, 1995a). Moreover, characterization of different neutralization-resistant escape mutant viruses with a panel of six anti-EAV neutralizing MAbs, as well as competitive binding assays, indicated that this linear immunodominant region of G_L comprises at least three interactive neutralizing epitopes (Balasuriya *et al.*, 1995a; Balasuriya *et al.*, 1997; Deregt *et al.*, 1994).

However, the amino-terminal ectodomain of EAV is considerably larger and more exposed than its counterpart in PRRSV and LDV. Furthermore, this region of GP_5 in PRRSV is highly glycosylated and could therefore be less immunogenic (Plagemann, personal communication). It appears that glycosylation may cover antigenic domains of the GP_5 protein and consequently protects it from destructive action of antibodies. By injecting the entire PRRSV particle to mice, several groups of investigators have isolated MAbs directed against structural viral proteins but none of these MAbs showed any reactivity with the GP_5 (Dea *et al.*, 1996; Drew *et al.*, 1995; Meulenberg *et al.*, 1997a; Nelson *et al.*, 1993; van Nieuwstad *et al.*, 1996; Wieczorek-Krohmer *et al.*, 1996). We therefore immunized mice with an ORF5 recombinant fusion protein expressed in *E. coli*, and succeeded to generate hybridomas secreting anti-GP_5 MAbs. Some subclones of these hybridomas secreted PRRSV neutralizing MAbs specific to the GP_5 (Part II, Chapter 1, Article 1). This part of our results demonstrated that the unglycosylated regions of the GP_5 are also implicated in neutralizing antigenic domains of the ORF5 encoded protein. The reactivity of the above mentioned MAbs with the native form of the viral protein in IIF tests as well as that of rabbit anti-ORF5 hyperimmune sera following immunization with unglycosylated form of the GP_5 expressed in *E. coli* confirms our previous results. We also established that as in other arteriviruses, the ORF5 encoded major envelope glycoprotein of PRRSV is the counterpart of G_L in EAV. However, due to great structural differences between the GP_5 of PRRSV and G_L of EAV, it seems unlikely that the neutralizing domains of these proteins are located at identical regions.

Interestingly, the MAbs reported in our work selectively reacted with the homologous PRRSV isolate and the US prototype strain ATCC VR-2332, but failed to react with the European prototype LV strain. This finding was suggestive of antigenic variability of the GP_5. As in most RNA viruses, notably EAV (Balasuriya *et al.*, 1995b; Glaser *et al.*, 1995; St-Laurent *et al.*, 1997), PRRSV is also genomically heterogenous and according to several reports, strain variations

occur in PRRSV (Andreyev *et al.*, 1997; Drew *et al.*, 1995 and 1997; Meng *et al.*, 1995a and b.; Suárez *et al.*, 1996b). All these reports indicate that the North American strains of PRRSV can be placed in a group distinct from that of the European strains, but strain variations also occur within the strains isolated from PRRS clinical cases of each continent. According to the reports of several groups of investigators, the ORF5 region of PRRSV is highly variable, resulting in a large number of aa substitutions in the GP_5 (Andreyev *et al.*, 1997; Meng *et al.*, 1995a; Murtaugh *et al.*, 1995). This prompted us to investigate if such variability also exists amongst the Canadian isolates (Part II, Chapter 2, Article2). The results of ORF5 nt sequencing data of nine Canadian isolates, compared with that of LV and the US MLV strain originated from ATCC VR-2332, indicate that while a certain degree of variability is associated with the Canadian isolates, they are more related to the two US prototype isolates but remarkably distant from the European LV prototype. Furthermore, the Québec field isolates can be included in a subgroup apart from the US and Ontario isolates. Our next objective was to establish if the genomic variability of ORF5 results in antigenic variability of the GP_5. A panel of anti-GP_5 MAbs and strain-specific rabbit anti-sera directed against recombinant ORF5 encoded proteins derived from five different PRRSV strains, comprising three Canadian isolates, the US MLV strain and the European LV strain, were included in this study. We established that the GP_5 plays a role in antigenic variability amongst different strains. Presumably, the antigenic diversity is the result of variability of the genomic region encoding the protein in question. However, the similarities in reactivity patterns of MAbs with viral proteins does not always follow the phylogenic relation between strains since a single aa mutation within an epitope may totally ablate the reactivity of a MAb with a protein. While rabbit strain-specific anti-ORF5 sera cross-reacted with the heterologous strains, highest IIF titers were obtained when each antisera was reacted with it's autologous strain. This can be due to the concomitant association of common and strain-specific epitopes with the GP_5 of the five strains of PRRSV used in our experiments. The shared epitopes are implicated in the cross-reactivity

of the strain-specific antisera while strain-specific epitopes result in higher titers when these antisera are tested with the autologous strains of the virus. Variation in titers of monospecific rabbit antisera may also be explained by the diminished affinity of the antibodies directed against a single epitope due to the point-mutation of the latter in different strains.

The genomic variability may be attributed to the lack of proofreading function of the viral RNA dependent RNA polymerase. This induces errors when copying the viral genome. Concurrence of variable regions with the hydrophilic domains of GP_5 can be the result of the host's selective antibody response directed against the exposed domains of this envelope glycoprotein which in turn favors antigenic drifts. According to the above mentioned studies, the GP_5 of PRRSV is the most variable structural protein of PRRSV and while this protein is associated with neutralizing epitopes, its high degree of variability may contribute to an effective mechanism for the emergence of antigenic variants and evading the host's immune surveillance. This may in part explain the mechanism of viral persistence.

Unlike EAV (Chirnside *et al.*, 1995a), immunizing pigs and rabbits with the *E. coli* expressed ORF5 does not trigger the immune system to produce neutralizing antibodies (Loemba *et al.*, 1996). This could be the result of structural differences existing between the two proteins or differences in functional immune systems of these unrelated animal species, making pig and rabbit tolerant to the linear epitopes of the *E. coli*-expressed ORF5 encoded recombinant protein. Therefore, DNA immunization was used to evaluate the immunogenicity of the native form of GP_5 in pigs.

While immunization by plasmidic DNA has been described in laboratory animals since 1980's, little experimental data is still available concerning the practical value of DNA vaccination in farm animals. More specifically, no DNA

immunization model was available in pigs when we started our research work in this area.

In these experiments, pigs and mice were injected with either a bacterial plasmid encoding the GP_5 under the control of the HCMV promoter, or the ORF5 encoded recombinant fusion protein expressed in *E. coli* (Part II, Chapter 3, Article 3). The results demonstrated that while both group of pigs respond to antigen inoculation by seroconversion, only DNA-immunized pigs develop neutralizing antibodies in response to ORF5 encoded GP_5 of PRRSV. Confirming our previous findings, BALB/c mice developed neutralizing antibodies in response to immunization with DNA encoding the GP_5 or the recombinant form of this protein expressed in *E. coli*. We therefore conclude that the GP_5 is sufficient to elicit neutralizing antibodies in pigs. This conclusion was further confirmed by our other study (Chapter 2, Article 4), showing the existence of a significant correlation between the neutralizing antibody titers present in PRRSV-convalescent sera and their ELISA titers with the ORF5-encoded protein. It appears that pigs recognize conformational epitopes of the GP_5 and develop neutralizing antibodies in response to correctly modified form of the GP_5 expressed in host cells. The anti-GP_5 specificity of the immune response was established by *ex vivo* proliferation of PBMC in presence of ORF5-encoded recombinant protein and the reactivity of the sera with GP_5 in WB, IIF and ELISA. Our results also showed that DNA-immunization with a plasmid encoding the GP_5 of PRRSV protects pigs from developing intensive PRRSV-induced lesions observed in unvaccinated, virus-challenged controls. Virus dissemination to organs other than the lungs and the accessory lymph nodes was not observed in DNA-immunized animals after a massive virus challenge, and these animals had remarkably lower virus burden in their respiratory system as compared to the GST-ORF5 immunized or unvaccinated controls. In these experiments, pigs immunized with the recombinant fusion protein developed an intensive interstitial pneumonitis following virus challenge. An explanation for these results could be the antibody

dependent enhancement (ADE) phenomenon which has been reported to occur in pigs having subneutralizing levels of antibodies to PRRSV (Yoon *et al.*, 1996 and 1997). On the contrary, virus replication is significantly inhibited in presence of neutralizing antibody titers. An alternative explanation of this results can be local secretion of pro-inflammatory cytokines in the lungs of GST-ORF5 immunized pigs.

So far, there is little published data in relation with the post-immunization cellular immune response of pigs to PRRSV. $CD4^+$ T cells are found to act as effector cells sensitive to viral antigen (Bautista & Molitor, 1997). By genetic immunization we have shown that cellular immune response specific to GP_5 is independent from, and it precedes the appearance of specific anti-PRRSV neutralizing antibodies. Antigen-specific blastogenesis in response to the recombinant ORF5-encoded protein suggests that cellular immune responses are indeed essential in protecting pigs against PRRSV infection. Identification of specific domains of GP_5, implicated in cellular immune response, may therefore be necessary for developing a recombinant subunit type vaccine.

Considering the high challenge dose used in our experiments, the protection conferred to the DNA-vaccinated pigs was quite remarkable. The only set back related to this method of immunization was persistence of PRRSV in the respiratory tract of virus challenge pigs which we believe is the result of the heavy virus challenge, which may not prevail in field conditions.

We found that DNA immunization is a safe, practical and economically feasible vaccination method in pigs that can be used in field, should application tools be available to practitioner at an affordable price. Unlike live-attenuated viral vaccines, this method of immunization does not have safety hazards such as tissue culture contaminants and pathogenesis in immunosuppressed recipients. Stress factors such as poor housing and over-crowding often prevails in intensive animal

raising operations, and can contribute to post-vaccination clinical outbreaks reported shortly after prophylactic immunization which is also common phenomenon in veterinary medicine. While DNA immunization induces a durable protection, the inoculated DNA is rapidly cleared from the organism. On the contrary, administration of live-modified vaccines may result in virus persistence which in turn, contributes to generation of mutants due to the selective immune pressure on the resident variants. Persistently-infected animals may eventually shed newly generated mutants, specially in case of unstable pathogens such as RNA viruses and cause new outbreaks. Production and purification of large quantities of viral particles, or their immunogenic structural proteins, for use in whole viral inactivated vaccines is economically unfeasible for low yield viruses such as PRRSV. Being the apparent choice for such pathogens, the recombinant type vaccines require precise experimental data relating to the viral immunodominant and neutralizing antigenic domains.

In general it appears that ORF5 may be a good candidate for a subunit recombinant type vaccine against PRRSV. However, the genomic variabilities of the ORF5 gene (Andreyev *et al.*, 1997; Meng *et al.*, 1995; Pirzadeh *et al.*, 1997) can be a major impediment in developing such vaccine. Further investigation is therefore needed to determine if genomic variability of ORF5 affects antigenic determinants of GP_5 involved in protection. The ADE phenomenon is also a potential problem for immunization against PRRSV (Yoon *et al.*, 1996 and 1997). Since it is suggested that different epitopes are involved in VN and ADE (Yoon *et al.*, 1997), it would be essential to establish if the GP_5 is indeed involved in ADE and the role of neutralizing epitopes of GP_5 in ADE.

Preliminary results of our epitope mapping experiments with truncated proteins indicated that PRRSV neutralizing convalescent sera recognize a region formed by 42 aa situated at the carboxy terminal of ORF5 encoded recombinant protein expressed in *E. coli* (Part II, Chapter 4). It has been demonstrated that pigs

exposed to PRRSV develop antibodies to the GP_5 as early as PID seven to eleven, whereas neutralizing antibodies are not detected any earlier than PID 21 to 28 (Loemba *et al.*, 1996). This suggests that some of the immunodominant epitopes of the GP_5 may not have a role in virus neutralization. This phenomenon may also be explained by general expansion of antibody secreting B cells in the beginning of immune response, when the circulating antibodies generally have a low affinity, followed by clonal selection of high affinity antibody secreting B cells towards the end of immune response. The reactivity pattern of neutralizing MAbs with the GP_5 is therefore essential in identifying neutralizing domains of GP_5.

In spite of major advances made in recent years relating to understanding of epidemiology, structural and molecular characteristics of PRRSV, some basic aspects of this infection, including the mechanism of viral pathogenesis and characterization of immune response of PRRSV-infected animals remain totally obscure. For example, abortion and other reproductive disorders are not explained by cell populations permissive to the virus and the organs that are targets of PRRSV lesions. The febrile syndrome alone, which is encountered in numerous other porcine diseases, cannot explain mummified fetus and delayed return to estrus. A plausible hypothesis could be the endocrine disorders that may result as a consequence of central nervous system lesions. While the answer to this question may lead to an effective symptomatic treatment of the disease, this aspect of PRRSV pathology has not yet been investigated. The mechanisms of animal immune response resulting in eliminating the infectious agent, or alternatively developing a persistent infection is another enigmatic aspect of PRRSV. As was described in detail, similar to retroviruses recognized as the causative agents of immunodeficiency syndrome in mammalians, PRRSV propagates in selective subpopulations of the immune cells, it can be transmitted through contaminated semen and it produces a persistant infection. Furthermore, as in HIV infection, PRRSV triggers apoptosis of the infected cells through expression of the GP_5 and during the acute phase of infection, a significant reduction in $CD4^+/CD8^+$ ratio is

observed. However, this reduction is not sufficient to induce an immunodeficiency in infected pigs at short terms. The long incubation period of immunodeficiency viruses generally exceeds the rearing period of farm animals. It is not clear if the PRRSV-infected pigs are reared long enough, they will develop an immunodeficiency-like syndrome. While this appears as a purely hypothetical question, it may have an important environmental impact, taking into consideration the newly emerged Arteriviruses which are implicated in animal epidemics. It is therefore important to identify the long term role of PRRSV in inducing an immunodeficiency syndrome.

PART IV

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Boroushan PIRZADEH

Publications and Communications:

Publications

Principal author:

- **Pirzadeh, B.** & Dea, S. (1997) Monoclonal Antibodies to the ORF5 Product of Porcine Reproductive and Respiratory Syndrome Virus Define Linear Neutralizing Determinants. *Journal of General Virology,* **78,** 1867-1873.

- **Pirzadeh, B.,** Gagnon, C.A. & Dea, S. (1997) Genomic Variation and Antigenic Variations of the ORF5 Genes of Porcine Reproductive and Respiratory Syndrome Virus Field Isolates. (Manuscript accepted for publication in *Canadian Journal of Veterinary Research.*

- **Pirzadeh, B.** & Dea, S. (1997) Immune Response in Pigs Following DNA Immunization with Plasmid DNA Encoding ORF5 of Porcine Reproductive and Respiratory Syndrome Virus. (Manuscript accepted for publication in *Journal of General Virology*).

Co-author:

- Gonin, P., **Pirzadeh, B.**, Gagnon, C.A. & Dea, S. (1997) Virus neutralization of Porcine Reproductive and Respiratory Syndrome Virus correlates with antibody response to the GP_5. (Manuscript accepted for publication in *Journal of Veterinary Diagnostics and Investigation*)

Communications:

- Induction of Antiviral Immune Response by DNA Vaccination with a Recombinant Plasmid Encoding major Envelope Glycoprotein of Porcine Reproductive and Respiratory Syndrome Virus. Poster at the 78th Conference of Research Workers in Animal Diseases; Nov 11-12, 1997 Chicago, USA.

- Induction of Antiviral Immune Response by DNA Vaccination with a Recombinant Plasmid Encoding major Envelope Glycoprotein of Porcine Reproductive and Respiratory Syndrome Virus. Oral Presentation at 16th annual meeting of American Society for Virology Bozeman, Montana, July 1997.

- Eukaryotic and Prokaryotic Expression of ORF3 to 7 of Porcine Reproductive and Respiratory Syndrome Virus. Oral presentation at 16th annual meeting of American Society for Virology Bozeman, Montana, July 1997.

- Evidence for a Specific Binding Factor for Porcine Reproductive and Respiratory Syndrome Virus. Oral presentation at 47th annual meeting of Canadian Society for Microbiology, Quebec, Canada, June 1997.

- Immunogenicity of the ORF5 Encoded E Glycoprotein of Porcine Reproductive and Respiratory Syndrome Virus. Oral presentation at 47th annual meeting of Canadian Society for Microbiology, Quebec, Canada, June 1997.

- Characterization of the Monoclonal Antibodies to the Glycoprotein Encoded by the ORF5 Gene of Porcine Reproductive and Respiratory Syndrome Virus. Oral presentation at the 77th Conference of Research Workers in Animal Diseases; Nov 11-12, 1996 Chicago, USA.

- Antigenic Variability of the Envelope Glycoprotein of Porcine Reproductive and Respiratory Syndrome Virus Defined by Monospecific Antisera and Monoclonal Antibodies. Poster at the 77th Conference of Research Workers in Animal Diseases; Nov 11-12, 1996 Chicago, USA.

- Genomic and Antigenic Variability of the Envelope Glycoprotein of Porcine Reproductive and Respiratory Syndrome Virus and it's Cross Reactivity with Monoclonal Antibodies."
Oral presentation (in French language) at the 11th Annual Conference of Animal Pathology, Oct. 9-10, 1996 St-Hyacinthe (Québec), Canada.

- Serological Studies of Variability and Epitope Mapping of the Envelope Glycoprotein of Porcine Reproductive and Respiratory Syndrome Virus. Oral presentation (in French language) at the "Journée de recherche et Colloque en Zootechnie, Sainte-Foy, May 30-31, 1996 (Québec) Canada.

Annex I :

The following is contribution of Boroushan Pirzadeh to articles listed in this thesis with at least one co-author other than the thesis director.

Article 2 entitled:

"Genomic and antigenic variations of porcine reproductive and respiratory syndrome virus."

Specific contribution:

Cloning of the ORF5 coding region of five different strains in pGEX, expression in *E. coli*, purification of the corresponding recombinant proteins, immunization of rabbits, collection and preparation of sera, performing all serological tests and preparation of the manuscript.

Article 4 entitled:

"Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to GP_5 major envelope glycoprotein."

Specific contribution:

Expression and purification of the recombinant ORF5 encoded protein of PRRSV in *E. coli*, development of an ELISA test with the above mentioned recombinant fusion protein, partipation in prepation of the manuscript.