SCREENING FOR POLYMORPHISM IN 2 CANDIDATE GENES OF GENERALIZED DEFENSE POWER IN PIGS : COMPLEMENT COMPONENT C5 AND INTERLEUKIN 12

Thesis

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K.GANESH KUMAR

Roll No. 513

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IZATNAGAR - 243 122 (U.P.) INDIA

2003

Dr. Sanjeev Kumar M.V.Sc., Ph.D.,
Senior scientist

Molecular Genetics Laboratory,
Central Avian Research Institute,
Izatnagar – 243122

Date: 24th October 2008

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Certified that the research work embodied in the thesis entitled '**Screening for polymorphism in 2 candidate genes of generalized defense power in pigs: complement component C5 and Interleukin 12**' submitted by **Dr. K. Ganesh Kumar**, Roll No. 513, for the award of Doctor of Philosophy degree in the discipline of **Animal Genetics** in Indian Veterinary Research Institute, is the original work carried out by the candidate himself under my supervision and guidance.

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Sanjeev Kumar

(Sanjeev Kumar)
Chariman
Student's advisory committee

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Signature of the external examiner
Date:---------22.1.2004

Dr. Sanjeev kumar
Chariman
Adivosry committee
Date:------24.10.03

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DEDICATED TO MY MOTHER

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(K. GANESH KUMAR)

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A	: Adenosine
ABTS	: 2,2′Azino bis (3-ethylbenzthiazo-line-6-sulphonic acid) diammonium salt
ACH	: Alternative complement activity
APS	: Ammonium peroxydisulphate
AR	: Autoregressive
ASP	: Acylation stimulation protein
ARH	: Heterogenous autoregressive
BCR	: B-cell receptor
bp	: Base pair
C	: Cytisine
°C	: Degree Celcius
C5	: Complement component C5
CD	: Cluster of differentiation
cDNA	: Complementary deoxy ribonucleic acid
CH	: Complement activity
CR	: Complement receptor
CS	: Compound symmetry
CSH	: Heterogenous compound symmetry
DAF	: Decay accelerating factor
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleotide triphosphate
DM	: Dystrophia myotonica locus
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DTT	: 1, 4, Dithio theritol

DUMI	: F2-Resource population, a cross of Duroc and Berlin miniature pigs
FAM	:
E.coli	: Escherichia coli
EDTA	: Ethylenediaminetetraacetic acid
ELISA	: Enzyme-linked immunosorbent assay
EST	: Expressed sequenced tag
FAO	: Food and Agriculture Organization of the United Nation
Fc	: Crystallizable fragment of immunoglobulin G molecule
FCS	: Fetal calf serum
G	: Guanine
HCl	: Hydrochloric acid
hr	: Hour
IFN-γ	: Interferon-gamma
IL	: Interleukin
IPTG	: Isopropyl -D- thiogalactopyranoside
Kb	: Kilobase
kDa	: Kilodalton
KIRs	: Killer cell inhibitory receptors
LPS	: Lipopolysaccharide
LRT	: Likelihood ratio test
MAC	: Membrane attack complex
MAS	: Marker assisted selection
MASPs	: MBL-associated proteases
MBL	: Mannose binding lectin
MCP	: Membrane cofactor protein
mg	: Milligram
MHC	: Major histocompatibility complex

min	: Minute
ml	: Milliliter
mRNA	: Messenger RNA
Mw	: Molecular weight
NaOH	: Soduim hydroxide
NK	: Natural killer
NRAMP1	: Natural resistance associated macrophage protein 1 gene
ns	: Not significant difference
OD	: Optical Density
ORF	: Open reading frame
PAMPs	: Pathogen associated molecular patterns
PBMCs	: Peripheral blood mononuclear cells
PCR	: Polymerase chain reaction
PCR-RFLP	: PCR-restriction fragment length polymorphism
PHA	: Phytohemaglutinin
PRRS	: Porcine reproductive and respiratory syndrome
QTL	: Quantitative trait loci
RACE-PCR	: Rapid amplification of cDNA ends-PCR
RCA	: Regulators of complement activition
RE	: Responsive element
RNA	: Ribonucleic acid
rpm	: Rotations per minute
SAS	: Statistical Analysis System software
SD	: Standard deviation of mean
SDS	: Sodium dodecyl sulfate
SLA	: Swine leukocyte antigens
SNPs	: Single nucleotide polymorphisms

SM	: Sodium chloride - magnesium sulphate buffer
SSCP	: Single strand conformation polymorphism
SSLP	: Simple sequence length polymorphism
T	: Thymidine
TBE	: Tris-Boric acid-EDTA buffer
TCRs	: T-cell receptors
TE	: Tris-EDTA buffer
TEMED	: N, N, N′, N′-Tetramethylendiamine
TNF-α	: Tumor necrosis factor –alpha
TGF	: Transforming growth factor
UTR	: Untranslated region
VIC	:
W	: Watt
w/v	: Weight by volume
X-gal	: 5-Bromo 4-chloro-3-indolyl-β-D-galactoside
µg	: Microgramm
µl	: Microliter

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1 Introduction

Since time immemorial, when man started domesticating animals, he knowingly or unknowingly started breeding them for the characters he required for. With the advancement of science and understanding of genetics, the genetic improvement of domestic animals was practiced, but the focus was more towards improvement of traits which were considered to be economically worthy by him. Little importance was given about the disease resistance ability of animals, since it was coped with alternative ways like good management, vaccines, drugs, quarantine, isolation and slaughtering of diseased animals. Moreover, man had selected animals in view of improving commercial products which had negative impact on disease resistance. Nevertheless, of late there was surge in the interest towards breeding for disease resistance traits because of various reasons like increasing resistance of pathogens against drugs, increasing awareness and hence resistance among peoples for using drugs and chemicals in agricultural products and finally also due to the economic potential of having disease resistant animals. Moreover, economic losses from disease of farm animals have been estimated to account for 10 – 20 % of the total production costs (Müller and Brem, 1991). Breeding for disease resistance is an alternative way to solve the problems of disease in farms in long term perspective and to reduce the costs of disease control.

To explore the genetic possibilities, one has to know the mechanism of how an animal protect itself against various pathogens. First protective measure is at the level of invasion of pathogen. Not many microorganisms can cross the physical barrier of mucous and serous layers. The second step is pathogen's ability, after successfully crossing the barrier, to establish a strong foothold to cause disease. This is regulated by hosts innate or acquired immune responses or, much more commonly, by a range of innate non-immunological factors, such as lack of essential nutrients/ substrates, lack of receptors for potential intracellular parasites, incompatible intracellular processing mechanisms, etc. The third level is pathogen can establish but is still unable to produce a disease. There is also a fourth level which is more of protection of a population than in individual level. The effect is most obvious in the case of the helminthiases and

other parasitic infections. In these examples, in a population, resistant individuals exert a controlling effect on levels of parasites and thus reduce challenge levels to the benefit of all members of the population, resistant or otherwise.

Of all the above levels, the least importance in geneticist's point of view is the first level i.e the level of physical barrier. At the second level, level of barriers to establishment of invading organisms, experiments in various species suggests that aspects of innate and acquired immunity may offer opportunities for exploitation of genetically determined variability. The immunological mechanisms involved in host protection against the array of pathogens, in evolutionary higher animals, are more complicated and evolutionary more complex, as they develop to cope up with the ever growing varieties of pathogens. These mechanisms differ between animals at genetic level. They are advantageous from geneticist's point of view because of their polymorphic nature.

A brief consideration of the general approaches which have been and are currently being taken in research on immune response and disease resistance in farm animals gives a view of what is to be done. The most basic studies have involved breed or population comparisons. Many studies have been reported, and those which have been carefully conducted and properly controlled to minimize environmental effects have led to identification of potentially valuable genetic resources. The continuation of the above study, using the resources developed, the search for disease-resistance markers has involved screening for associations between disease resistance and a variety of polymorphisms, often in candidate genes or in QTL regions. The MHC has understandably received considerable attention in this regard in many species. The present study is carried out to contribute to the wealth of information pouring into this field. In this study, two genes complement component *C5* and interleukin 12 (*IL 12*) were considered as candidate genes for disease resistance in pigs.

It is now an established fact that cytokines and complement system play a very vital role in protecting animals against invading organisms. The complement system is a potent unspecific mechanism of host defense. The complement component *C5* being

the major protein of the terminal sequence of the complement cascade and was not studied much in porcine species. Interleukin 12 is the main activator of NK cells and induces interferon gamma production when encountered with microbes, i.e. it is important in non-specific immune defense, the study of which is also not done much in pigs. *C5* and *IL 12* represent promising candidate genes for generalized defense power of pigs. Hence, the present study was planned with the following **objectives.**

1. Detection of single nucleotide polymorphisms (SNPs) in porcine complement component *C5* and Interleukin 12 genes in pigs and

2. Large-scale association analysis for disease resistance using the SNPs detected.

2 Review of literature

2.1 Immune system

The human/ animal body encounters foreign material from the environment constantly. The first barriers skin and mucosal surfaces need to protect the body against invasion by microbes, foreign antigens and toxic agents. The immune system should recognize and remove all foreign material but spare the body's own viable tissues. Moreever, the immune system should also be able to recognize non-viable tissues as it is responsible for the clearance of tissue debris generated during trauma or during the normal cellular turn-over.

The first response of the body to pathogens, which crosses the physical barrier of skin and mucous, is an inflammatory reaction which includes migration of cell types having defensive functions, alterations in vascular permeability and the secretion of soluble mediators, such as cytokines, chemokines and interferons (IFNs). The inflammatory cells and soluble mediators have potent antimicrobial activity and together they represent the effector phase of innate immunity. Innate immunity refers to antigen-nonspecific defense mechanisms that a host uses immediately or within several hours after exposure to an antigen. This is the immunity one is born with.

Unlike adaptive immunity, innate immunity does not recognize every possible antigen. Instead, it is designed to recognize a few highly conserved structures present in many different microorganisms. The structures recognized are called pathogen-associated molecular patterns (PAMP) which includes lipopolysaccharides (LPS) from the gram-negative cell wall, peptidoglycan, lipotechoic acids from the gram-positive cell wall, the sugar mannose (common in microbial carbohydrates but rare in those of humans), bacterial DNA, double-stranded RNA from viruses, and glucans from fungal cell walls. Most body defense cells have pattern-recognition receptors for these common PAMPs and so there is an immediate response against the invading microorganism. The receptors includes β2 integrin CR3 or ITGB2 that are broad range pattern recognition receptors (Ehlers, 2000), the family of Toll-like receptors (TLRs) is

composed of at least ten members, which are distributed differentially between inflammatory cells, plays an important role. These receptors recognize different classes of pathogen's and induce the production of similar, but not identical, patterns of pro-inflammatory mediators. The distribution of the receptors on inflammatory cell types determines the characteristic patterns of cytokines, chemokines and IFNs induced by different pathogens, which are largely responsible for the cellular and vascular inflammatory reactions, as well as the activation of innate resistance. PAMPs can also be recognized by a series of proteins in the blood that initiate the complement pathways.

Although innate resistance is efficient at either preventing an infection or greatly reducing the pathogen load, sterile cure or control of an infection is achieved only when adaptive immunity is induced. However, because of the need for expansion of antigen specific T and B cell populations with clonally distributed receptors, an efficient adaptive response is induced only approximately a week after a primary infection. Innate resistance and adaptive immunity are not simply sequential and complementary mechanisms of resistance to pathogens – they regulate each other, through cellular contacts and the secretion of soluble mediators. In particular, the cytokine milieu that is established during the inflammatory innate response to pathogens sets the stage for the migration of antigen specific T cells to lymph nodes, where they meet antigen presenting cells (APCs).

T helper 1 or T helper 2 (Th1 or Th2) responses, which are effective against intracellular and extra cellular pathogens, respectively, are then induced, depending on the pattern of cytokines present during the clonal expansion of antigen specific T cells (Mosmann and Coffman, 1989). The cytokines produced during the inflammatory innate response direct the deviation of T cells towards either type of adaptive effector response. Interleukin-4 (IL-4) was identified first as a cytokine that is instrumental in the generation of Th2 responses and later *IL 12* was discovered and recognized to have a central role in Th1 responses.

Moreover, there are circulating proteins, in blood system, in inactive form but in response to the recognition of molecular components of microorganism, they become sequentially activated, working in a cascade wherein the binding of one protein promotes the binding of the next protein in the cascade, these proteins together represents the complement system, which not only work to eliminate the invading organisms but are also helpful in the activation of specific immune response against them.

This chapter is focused on review of Interleukin 12 and complement system especially on complement component *C5* and their role in immunity.

2.1.1 Interleukin 12 (*IL 12*)

Interleukin 12 (*IL 12*) was identified as a product of Epstein - Barr virus (EBV) – transformed human B cell lines that can activate NK cells, generate lymphokine activated killer cells (LAKs) and induce IFN-γ production and T-cell proliferation. (Stern *et al.*, 1990). *IL 12* is a heterodimeric cytokine consisting of glycosylated subunits of 35 and 40 kDa proteins linked by disulfide bonds (Podlaski *et al.*, 1992). These two subunits were produced by two unrelated genes located in two different chromosomes. It was originally described as CTL maturation factor (TCMF) (Gately *et al.*, 1986), T cell stimulating factor (TSF) (Germann *et al.*, 1987), natural killer cell stimulatory factory (NKSF) (Kobayashi *et al.*, 1989) and as cytotoxic lymphocyte maturation factor (CLMF) (Stern *et al.*, 1990). *IL 12* is secreted by antigen-presenting cells, primarily macrophages, interacting with bacteria or bacterial products, such as lipopolysaccharide (LPS) (Gubler *et al.*, 1991; Baron *et al.*, 1993; D' Andrea *et al.*, 1993; Hsieh *et al.*, 1993).

The induction of *IL 12* during the early stages of the immune response affects both the innate and antigen specific responses. *IL 12* enhances innate immunity through the induction of IFN-γ expression (Wu *et al.*, 1993), enhanced cytotoxicity (Cesano *et al.*, 1993; Asteamezaga *et al.*, 1994) and proliferation of NK cells (Gately *et al.*, 1991). *IL 12* affects the antigen specific immune responses by stimulating proliferation of

antigen stimulated T- cells (Zeh *et al.,* 1993) alone and synergistically with *IL 2* and by increasing the cytotoxicity of CD8+ lymphocytes (Mehrotra *et al.,* 1993). The presence of *IL 12* during the initial priming of naive helper T-cells induce a Th-1 type cytokine response in those cells (Hsieh *et al.,* 1993) and serves as a second signal to induce proliferation and cytokine secretion of Th1 clones but not Th 2 clones (Macatonia *et al.,* 1993; Yanagida *et al.,* 1994).

An IL 12 receptor (IL 12R) is expressed on phytohaemagglutinin (PHA) activated lymphoblasts and is present on activated CD 4+ and CD 8+ T-cells and activated NK cells but not on resting or activated B-cells (Desai *et al.,* 1992). Alpha subunit of the IL 12 receptor (IL 12R α) homologous to group 130 was cloned and identified as a possible signal transduction molecule (Chua *et al.,* 1994). Although active *IL 12* is a heterodimer, the two subunits are regulated independently. The 40 kDa subunit or a homodimer of the *p40* subunit may act as an antagonist to the effect for the heterodimeric form of the cytokine (Mattner *et al.,* 1993). *In vivo* studies have supported the importance of *IL 12* effects on both innate and specific immunity in the generation of protective immunity. In a study, SCID and normal mice were infected with *Listeria* sp. After injecting neutralizing antibodies to *IL 12*, both suffered increased mortality to infections (Tripp *et al.,* 1994). In another study it was reported that normally susceptible strains were cured and made durably resistant to Leishmania infections with treatments with recombinant *IL 12* for the first 7 days of infection (Heinzel *et al.,* 1993). In contrast, mice treated with *IL 12* have a more severe disease than untreated mice when infected with a nematode parasite, *Nippostronglylus brasiliensis* (Finkelman *et al.,* 1994). The above studies implied the importance of *IL 12* in immune function of animals.

2.1.1.1 Structure of *IL 12*

Among the family of interleukin proteins characterized to date, *IL 12* has a novel structure. It is a heterodimeric protein comprised of two disulfide linked subunits designated *p35* and *p40*, which are encoded by unrelated genes. The genes for the *IL 12* subunits *p35* and *p40* residue are located at independent loci in the porcine

genomes on chromosomes 16 and 13, respectively. The genes encoding the human *IL 12p35* and *IL 12p40* subunits have been localized on chromosomes 3 and 5, respectively.

No sequence homology exists between the *IL 12p35* and *p40* subunits. However, the 35-kDa subunit of *IL 12* shares homology with *IL 6*, G-CSF, and chicken myelomonocytic growth factor (Merberg *et al.*, 1992) and has similar to many other cytokines, an α-helix rich structure. Interestingly, the 40 kDa subunit is not homologous to other cytokines, but belongs to the hemopoietin receptor family, and most resembles the extra cellular domain of the *IL 6* receptor α-subunit and the ciliary neurotrophic factor receptor (Schoenhaut *et al.*, 1992; Gearing *et al.*, 1991). However, no evidence has appeared for the existence of membrane associated forms of *IL 12* or either of its subunits. The unusual structure of *IL 12* might have evolved from a primordial cytokine of the *IL 6* family and one of its receptors. *IL 23* and *IL 27*, two other heterodimeric cytokines that are related to *IL 12* have been identified recently, which indicates that *IL 12* is the prototype member of a small family of heterodimeric cytokines.

Neither *IL 12* subunit alone was found to display significant biological activity over a large range of concentrations (Gubler *et al.*, 1991; Wolf *et al.*, 1991) although a *p40* homodimer $(p40)_2$ may function as an *IL 12* antagonist that binds to the *IL 12*R but does not mediate a biologic response (Ling *et al.*, 1995; Gately *et al.*, 1996). Production of *IL 12* both by cell lines and by normal monocytes results in the secretion of a 5 to 500 fold excess of *p40* relative to the *IL 12* heterodimer (D' Andrea *et al.*, 1992; Podlaski *et al.*, 1992).

2.1.1.2 Biological activities of *IL 12*

Regulation of Th1/Th2 Responses by *IL 12*.

The most important of *IL 12* activities is its ability to regulate the balance between Th1 and Th2 cells. Th1 cells secrete *IL 2* and IFN-γ, thus promoting cell-mediated

immunity, whereas Th2 cells produce *IL 4, 5, 6, 10* and *13*, thereby enhancing humoral immune response (Scott , 1993; Paul and Seder, 1994). Studies carried out in humans (Paul and Seder, 1994; Wu *et al.,* 1993) and murines (Hsieh *et al.,* 1993; Seder *et al.,* 1993; Schmitt *et al.,* 1994) showed that *IL 12* promotes Th1 responses. *IL 12* does this in three ways; (a) it promotes the differentiation of naive T cells, during initial encounter with an antigen, into a population of Th1 cells capable of producing large amounts of IFN-γ following activation (Hsieh *et al.,* 1993; Seder *et al.,* 1993; Schmitt *et al.,* 1994). (b) it serves as a co-stimulus required for maximum secretion of IFN-γ by differentiated Th1 cells responding to specific antigen (Murphy *et al.,* 1994; DeKruyff *et al.,* 1995) and (c) it stimulates the development of IFN-γ producing Th1 cells from populations of resting memory T cells interacting with an antigen to which they have been previously exposed (Wu *et al.,* 1993; Paul and Seder, 1994; DeKruyff *et al.,* 1995). Although *IL 12* has been most commonly found to promote Th1 responses while suppressing Th2 responses, under some experimental conditions *IL 12* was observed to enhance Th2 responses (Schmitt *et al.,* 1994; Shu *et al.,* 1994; Wang *et al.,* 1994; Wynn *et al.,* 1995). Whether *IL 12* suppresses or enhances Th2 responses appears to depend both on the cytokine milieu and on the maturational state of the T cells (Gately *et al.,* 1998).

Induction of Cytokines secretion by *IL 12.*

One of the most important properties of *IL 12* is its ability to induce the production of large amounts of IFN-γ from resting and activated T and NK cells (Kobayashi *et al.,* 1989; Desai *et al.,* 1992; Gazzinelli *et al.,* 1993). This activity of *IL 12* is central to many of the effects seen when *IL 12* is administered *in vivo* (Heinzel *et al.,* 1993; Nastala *et al.,* 1994) and provides a mechanism whereby *IL 12* plays an important role in innate, as well as adaptive immunity. *IL 12* induces the production of several cytokines in low amounts, including TNF-α (Aste-Amezaga *et al.,* 1994; Nastala *et al.,* 1994), induction of cytokines and cytotoxic cell-associated molecules in human T and NK cells (Aste-Amezaga *et al.,* 1994; Naume *et al.,* 1993), *IL 2* (Perussia *et al.,* 1992) and IL 8 (Naume *et al.,* 1993). It was found that *IL 12* induces *IL 10* production

in *in vivo* administered mice (Morris *et al.,* 1994), which showed that *IL 12* limits its own effects by negative feed back mechanism as *IL 10* is a cytokine known for its function to inhibit *IL 12* production. In another study *IL 12* is reported to suppress the production of TGF-α via a mechanism that is least partially IFN-γ dependent (Marth *et al.,* 1996; 1997).

Effects of *IL 12* on Antibody Responses.

IL 12 can either enhance or inhibit humoral immunity, depending on the Ig isotype and the stimulus to antibody formation (Gately *et al.,* 1998). In mice, administration of *IL 12* results in enhancement of IgG 2a, IgG 2b, and IgG 3 antibody responses by as much as 10 to 1000 fold (Buchanan *et al.,* 1995; Germann *et al.,* 1995), but IgG 1 antibody response was found to be suppressed with *IL 12* administration (McKnight *et al.,* 1994; Buchanan *et al.,* 1995). It was shown to inhibit IgE responses in a variety of experimental models both *in vitro* (Kiniwa *et al.,* 1992) and i*n vivo* (Germann *et al.,* 1995).

Other activities of *IL 12.*

IL 12 was found to enhance the lytic activity of NK and lymphokine activated killer cells (Kobayashi *et al.,* 1989; Stern *et al.,* 1990), promote specific cytolytic T lymphocyte responses (Gately *et al.,* 1992), act as a short-term growth factor for activated T and NK cells (Kobayashi *et al.,* 1989; Stern *et al.,* 1990; Robertson *et al.,* 1992) and synergize with stem cell factor with some of the other colony stimulating factors to induce the proliferation and differentiation of haematopoietic stem cells (Jacobsen *et al.,* 1993; Hirayama *et al.,* 1994). Increased levels of *IL 12* were correlated to different disease states of multiple sclerosis.

2.1.1.3 Genetic polymorphism in *IL 12* and its effect on phenotype

IL 12 polymorphism at nucleotide level has its effect on the phenotype. *IL 12* β gene in humans have 4 mutations (4475 – 4474delG, 1188A>C, Glu186Asp and Ser226Asn).

Transmission disequillibrium tests in families identified through children with atopic asthma or allergic rhinitis was conducted. Frequencies of Ser226Asn and 1188 A-C alleles in the parents were found to be 0.04 and 0.5, respectively and preferential transmission of the two alleles to asthma-affected or rhinitis affected children were not observed and was not associated significantly with total serum IgE level. These results indicated that polymorphisms in *IL 12β* are not likely to be associated with the development of atopy- related phenotypes (Noguchi *et al.*, 2001).

In contrast, *IL 12*β promoter heterozyogsity contributes to asthma severity in children, the data of which suggested that polymorphism in promoter region had significant association with asthama severity rather than asthma susceptibility (Morahan *et al.*, 2002).

A three year old female patient was found to be *IL 12* deficient and was associated with recurrent episodes of pneumococcal pneumonia with sepsis and other infections in the absence of fevers (Haraguchi *et al.*, 1998). *IL 12* deficiency in a child was found to be associated with *Salmonella* enteritis disseminated infection (Altare *et al.*, 1998).

A new disease susceptibility locus, *IDDM1*8, was found to be located near the interleukin 12 (*IL 12*) *p40* gene, significant bias in transmission of *IL 12* β alleles was observed in affected sibpairs and was confirmed in an independent cohort of simplex families. A single base change in the 3′ untranslated region (UTR) showed strong linkage disequilibrium with the T1D susceptibility locus. The *IL 12* β 3′ UTR alleles showed different levels of expression in cell lines. Hence it was concluded that variation in *IL 12 p40* production may influence T-cell responses crucial for either mediating or protecting against this and other autoimmune diseases (Morahan *et al.*, 2001).

A genome wide scan for QTL in Japanese families, for mite-sensitive asthma in a child was found to have a significant linkage to chromosome 5q31-q33 near the interleukin 12-β locus (Yolouche *et al.*, 2000). Hall *et al.* (2000) identified a putative polymorphic

region of the *IL 12 p40* gene. Position 1188 in the 3' UTR is polymorphic with the frequency of the common allele around 80 % in healthy UK Caucasoids. PCR genotyping of multiple Caucasoid groups and an African group has shown significant population variation. Moreover, in a case-control study it was found that the polymorphism was not associated with rheumatoid arthritis, Felty's syndrome or large granular lymphocyte syndrome with arthritis or multiple sclerosis and a nonsignificant increase in the B allele frequency was observed in the rare large granular lymphocyte syndrome without arthritis (odds ratio 2.02, 95 % CI 0.95-4.3).

Huang *et al.*, 2000 carried out polymorphism study in complete *IL 12 p40* gene in humans and found 11 polymorphic regions, but only in intronic regions. They concluded that dearth in polymorphism in coding regions indicated a high level of conservation of the gene.

2.2 The complement system

The complement system is a highly regulated and complex set of interacting proteins in blood plasma and on cell surfaces that can directly recognize, binds to and kill or remove invading microbes. The proteins circulate in an inactive form, but, they become sequentially actived in response to the recognition of molecular components of microorganism.

There are 3 complement pathways that make up the complement system: the classical complement pathway, the lectin pathway and the alternative complement pathway. The pathways differ in the manner in which they are activated and ultimately produce a key enzyme called C3 convertase. The classical complement pathway is activated by antigen-antibody complexes. The lectin pathway is activated by the interaction of microbial carbohydrates with mannose-binding proteins in the plasma and tissue fluids and the alternative complement pathway is activated by microbial cell walls. The end results of each pathway, however, are the same. All complement pathways carry out 5 beneficial innate defense functions which are briefed below.

a. Triggering inflammation

C5a, a subunit of complement component C5 is the most potent complement protein triggering inflammation. It causes mast cells to release vasodilators such as histamine so that blood vessels become more permeable, it increases the expression of adhesion molecules on leukocytes and the vascular endothelium so that leukocytes can squeeze out of the blood vessels and enter the tissue (diapedesis), it causes neutrophils to release toxic oxygen radicals for extracellular killing and it also induces fever. To a lesser extent C3a and C4a also promote inflammation.

b. Chemotactically attracting phagocytes to the infection site

C5a also functions as a chemoattractant for phagocytes. Phagocytes will move towards increasing concentrations of C5a and subsequently attach, via their CR1 receptors to the C3b molecules attached to the antigen.

c. Promoting the attachment of antigens to phagocytes (enhanced attachment or opsonization)

C3b and to a lesser extent, C4b can function as opsonins i.e. they can attach antigens to phagocytes. One portion of the C3b binds to proteins and polysaccharides on microbial surfaces, another portion attaches to CR1 receptors on phagocytes, B-lymphocytes, and dendritic cells for enhanced phagocytosis. Actually, C3b molecule can efficiently bind to any protein or polysaccharide. Human cells, however, produce Factor H that binds to C3b and allows Factor I to inactivate the C3b. On the other hand, substances such as LPS on bacterial cells facilitate the binding of Factor B to C3b and this protects the C3b from inactivation by Factor I. In this way, C3b does not interact with our own cells but is able to interact with microbial cells. C3a and C5a increase the expression of C3b receptors on phagocytes and increase their metabolic activity.

d. Lysis of gram-negative bacteria and human cells displaying foreign epitopes

Complement system helps to destroy gram-negative bacteria as well as human cells displaying foreign antigens (virus-infected cells, tumor cells, etc.) by causing their lysis. It can also damage the envelope of enveloped viruses.

e. Removing harmful immune complexes from the body

C3b and to a lesser extent, C4b help to remove harmful immune complexes from the body. They attach the immune complexes to CR1 receptors on erythrocytes which then deliver the complexes to fixed macrophages within the spleen and liver for destruction. Immune complexes can lead to a harmful Type III hypersensitivity.

2.2.1 The classical complement pathway

Although at least 21 different serum proteins have so far been identified as part of the classical complement pathway, one can look at it as a pathway that is primarily activated by either IgG or IgM binding to an antigen and involves 11 major serum protein components. The "tips" of the antibody (the F_{ab} portion) have shapes that are complementary to epitopes - portions of microbial proteins and glycoproteins found on the surface of the microbe. The F_c portion of IgG and IgM can activate the classical complement pathway by enabling the first enzyme in the pathway, C1, to assemble. Typically to activate the classical complement pathway, IgG or IgM is made in response to an antigen. The F_{ab} portion of IgG (2 molecules) or IgM (1 molecule) reacts with epitopes of that antigen. A protein called C1q first binds to the Fc portion of antigen-bound IgG or IgM after which C1r and C1s attach to form C1, the first enzyme of the pathway. The activated C1 now enzymatically cleaves C4 into C4a and C4b. The C4b then binds to adjacent proteins and carbohydrates on the surface of the antigen and then binds C2. The activated C1 cleaves C2 into C2a and C2b forming C4b2a, the C3 convertase. Now the classical complement pathway is activated. C3 convertase can now cleave hundreds of molecules of C3 into C3a and C3b. Some molecules of C3b bind to C4b2a, the C3 convertase, to form C4b2a3b, a C5 convertase

that cleaves C5 into C5a and C5b. C5b binds to the surface of the target cell and subsequently binds C6, C7, C8, and a number of monomers of C9 to form $C5b6789_n$, the Membrane Attack Complex (MAC).

2.2.2 The lectin pathway

The lectin pathway is mediated by mannan-binding lectin, also known as mannan-binding protein (MBP). MBP is a protein that binds to the mannose groups found in many microbial carbohydrates but not usually found in the carbohydrates of hosts. The MBP is equivalent to C1q in the classical complement pathway. Activation of the lectin pathway begins when MBP binds to the mannose groups of microbial carbohydrates. Two more lectin pathway proteins called MASP1 and MASP2 (equivalent to C1r and C1s of the classical pathway) now bind to the MBP. This forms an enzyme similar to C1 of the classical complement pathway that is able to cleave C4 and C2 to form C4b2a, the C3 convertase capable of enzymatically splitting hundreds of molecules of C3 into C3a and C3b. Then the MAC is formed in the same way as in classical complement pathway mentioned above.

2.2.3 The alternative complement pathway

The alternative complement pathway is mediated by C3b, either from the classical or lectin pathways or from C3 hydrolysis by water (Water can hydrolize C3 and form C3i, a molecule that functions in a manner similar to C3b). Activation of the alternative complement pathway begins when C3b (or C3i) binds to the cell wall and other surface components of microbes. Alternative pathway protein Factor B then combines with the cell-bound C3b to form C3bB. Factor D then splits the bound Factor B into Bb and Ba, forming C3bBb. A serum protein called properdin then binds to the Bb to form C3bBbP that functions as a C3 convertase capable of enzymatically splitting hundreds of molecules of C3 into C3a and C3b. The alternative complement pathway is now activated. Some of the C3b subsequently binds to some of the C3bBb to form C3bBb3b, a *C5* convertase capable of splitting molecules of *C5* into *C5*a and *C5*b. MAC formation takes place in the same manner as mentioned in section 2.2.1

2.2.4 Complement component *C5*

2.2.4.1 Structure of *C5* gene

The fifth component of complement C5 is a 190 kDa glycoprotein comprised of two disulfide linked polypeptide chains, α and β, with molecular mass of 115 and 75 kDa, respectively. *C5* mRNA of human was predicted to encode a 1676 amino acid promoelcule that contains an 18 amino acid leader peptide and a 4 amino acid linker seperating the α and β chains. The structure of C5 protein is given in fig. 1.

C5 is a member of the thioester-containing protein family including C3, C4 and α_2-macroglobulin (α_2M). In the human genome, *C3, C4* and *C5* are paralogous genes that are postulated to be generated by at least two rounds of genome-wide gene duplication at early stages of the vertebrate lineage (Kasahara 1998). The order of *C3/C4/C5* divergence, however, needs further analysis, although Hughes (1994) has postulated that *C5* diverged first, on the basis of a limited number of partial sequences available for the C3/C4/C5 family. The complete primary structure of C5 has been determined in humans and mice. In lower vertebrates, C5 protein composed of α and β-chains has been purified from the rainbow trout (*Onchorhyncus mykiss*) and the gilthead sea bream (*Sparus aurata*) (Nonaka *et al.*, 1981; Sunyer *et al.*, 1997; Franchini *et al.*, 2001). A C5b protein incorporated into the MAC was identified in carp (Nakao *et al.*, 1996). At the DNA level, a partial cDNA sequence encoding *C5* has recently been reported for trout (Franchini *et al.*, 2001). The *C5* structural gene is on chromosome 2 in mice (D'Eustachio *et al.*, 1986), chromosome 9q34 in humans (Wetsel *et al.*, 1988) and chromosome 1q18 or 1q213 in pigs (Ponsuksili *et al.*, 2001).

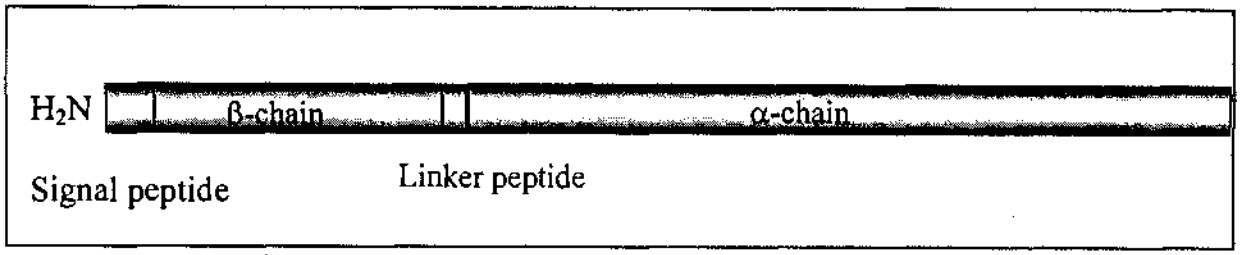


Fig. 1. Structure of the 5th component of complement protein.

2.2.4.2 Biologcial activity of C5

The fifth component of complement C5 is an important participant in inflammatory and cytolytic processes. Sera from C5 deficient individuals were found to lack bactericidal activity and have impaired ability to induce chemotaxis (Miller and Nilson, 1970). C5 is biosynthesized in hepatocytes (Patel and Minta, 1979), macrophages (Ooi and Calten, 1979), lung tissue (Wheat *et al.,* 1987) and type II alveolar epithelial cells (Strunk *et al.,* 1988) as an intracellular single chain precursor, pro-*C5*. It has been established that human pro-*C5* is synthesized as βα-chain orientation (Lundwall *et al.,* 1985). The pro-molecule is processed, glycosylated and secreted as a 190 kDa glycoprotein. Activation of any of the pathways leads to the formation of enzyme complexes, termed *C5* convertases, which cleave C5 into a small fragment, C5a, and a larger one, C5b. The smaller C5a fragment (C5a anaphylatoxin) is a cationic peptide derived from amino acid residues 1 – 74 of the C5 subunit and in humans, is cleaved off behind the arginine at position 74 by the C5 convertase with a trypsin-like substrate specificity (Fernandez and Hugli, 1978). C5a is a potent spasmogenic and chemotactic anaphylatoxin that mediates inflammatory responses at the site of infection by interacting with the G protein-coupled C5a receptor, which is expressed on the surfaces of myeloid and non-myeloid leukocytes (Zwirner *et al.,* 1999). The amino acid sequences of C5a in human (Fernandez and Hugli, 1978) swine, mouse (Gerhard and Hugli, 1980), bovine (Gennaro *et al.,* 1986) and rat (Cui *et al.,* 1994) have been determined. It is the most potent of the complement derived anaphylatoxins, expresses several biological activities including (a) contraction of smooth muscle (Cochrane and Müller-Eberhard, 1968), (b) degranulation of mast cells (Johnson *et al.,* 1975) and (c) chemotaxis of polymorphonuclear neutrophils (Goldstein and Weissman, 1974). On the other hand, the larger macromolecular product *C5*b, initiates the terminal part of complement activation, termed the lytic pathway, leading to formation of the membrane attack complex (MAC), or C5b-9 complex, which is responsible for the lysis of target cells. Thus C5 plays a crucial role in elimination of invading pathogens.

2.2.4.3 Deficiency of complement component C5

Several C5 deficient families have been reported in humans (Rosenfeld *et al.*, 1976, Snyderman *et al.*, 1979). Sera from homozygous C5 deficient individuals lack bactericidal activity and have a severely impaired ability to induce chemotaxis (Rosenfeld *et al.*, 1976). In addition, C5 deficient individuals display a propensity for severe recurrent infections particualarly to Neisserial species, including meningitis and extragenital gonorrhea (Snyderman *et al.*, 1979; Peter *et al.*, 1981). C5 deficiency in the mouse was one of the first complement deficiencies described. It was found that sera from the DBA/2 and B10.D2/Sn strains were hemolytically inactive which was identified to be C5 (Nilsson and mueller eberhard, 1967).

2.3 Breeding for disease resistance

The field of genetic disesase resistance, the attention for which is increasing nowadays, has been driven by two main criteria. The first of these, as with all scientific endeavour, is to increase knowledge and understanding. The second major stimulus to research in disease resistance is the prospect of its future commercial values. Now, it is a known fact that, there exists significant variation in farm animals in terms of resistance to diseases, and this variation is of economic importance. Nevertheless, the field has been slow to develop for several reasons. First, there are alternative options for disease control, such as management changes, treatment, vaccination, vector control, movement control, test and slaughter and isolation and quarantine. Second, the genetic route to improve the disease resistance of meaningful numbers of animals seems to be a slow and arduous one, due to the long generation times involved. Third, the spectre of negative impacts of improving disease resistance on valuable productivity traits is often shown to be a major hurdle, and fourth, the experimentation in disease resistance in farm animals can pose a considerable challenge, mainly because of the nature of the animal, which is expensive to obtain and maintain and relatively slow to reproduce. Nevertheless, of late there was surge in the interest towards breeding for disease resistance traits because of various reasons like increasing resistance of pathogens against drugs, increasing awareness and hence

resistance among peoples for using drugs and chemicals in agricultural products and finally also due to the economic potential of having disease resistant animals.

Genetic improvement for disease resistance in farm animals can be approached in two possible ways namely general and specific disease resistance. The latter approach is based on the assumption that a major gene or a single gene controls key resistance and susceptiblity for each possible disease, wheras the former approach is based on the acknowledgement of the polygeneic nature of resistance or susceptiblity related traits involving host response to pathogens. The major problem in both the approach is the difficulty in measuring the disease or the health status in animals. Since, infectious diseases are more complex as they involve variation in pathogen, host, host response and environment and hence making quantitative assessment difficult. Opportunity to observe infectious disease in breeding animals is very limited since management practices are designed to reduce disease incidence, and occurrence of any given disease is unpredictable. Inducing infection in enough individuals to provide meaningful data is not feasible. To adequately observe susceptibility and resistance phenotypes, the average disease incidence should be at least 50 %, although with leptospirosis occurring at 20 to 30 % over a 4-year period it was possible to select for resistant pigs (Pryztulski and Porzeczkowska, 1980).

Alternative strategies for genetically enhancing disease resistance might involve indirect, multitrait selection for candidate phenotypes and genotypes reflecting variation in host resistance-mediating functions, such as innate and immune response (Gavora and Spencer, 1983; Mallard *et al.*, 1998; Wilkie *et al.*, 1998). By this or other approaches, general improvement in resistance to infectious diseases may be achieved in high-producing livestock to derive substrate populations in which to delete or add disease-specific susceptibility or resistance alleles. Alternative strategies for optimizing health in livestock may involve methods for improving general disease resistance is by selecting the phenotypes associated with high performance of traits mediating host innate and immunologically specific effector functions. Disease-specific approaches are most likely to be relevant to monogenic traits for which the susceptibility phenotype can be controlled by avoiding the related alleles. Both

approaches require optimal management of the environment, and animals improved for general disease resistance are expected to be ideally suited for additional enhancement by deletion or addition of genes.

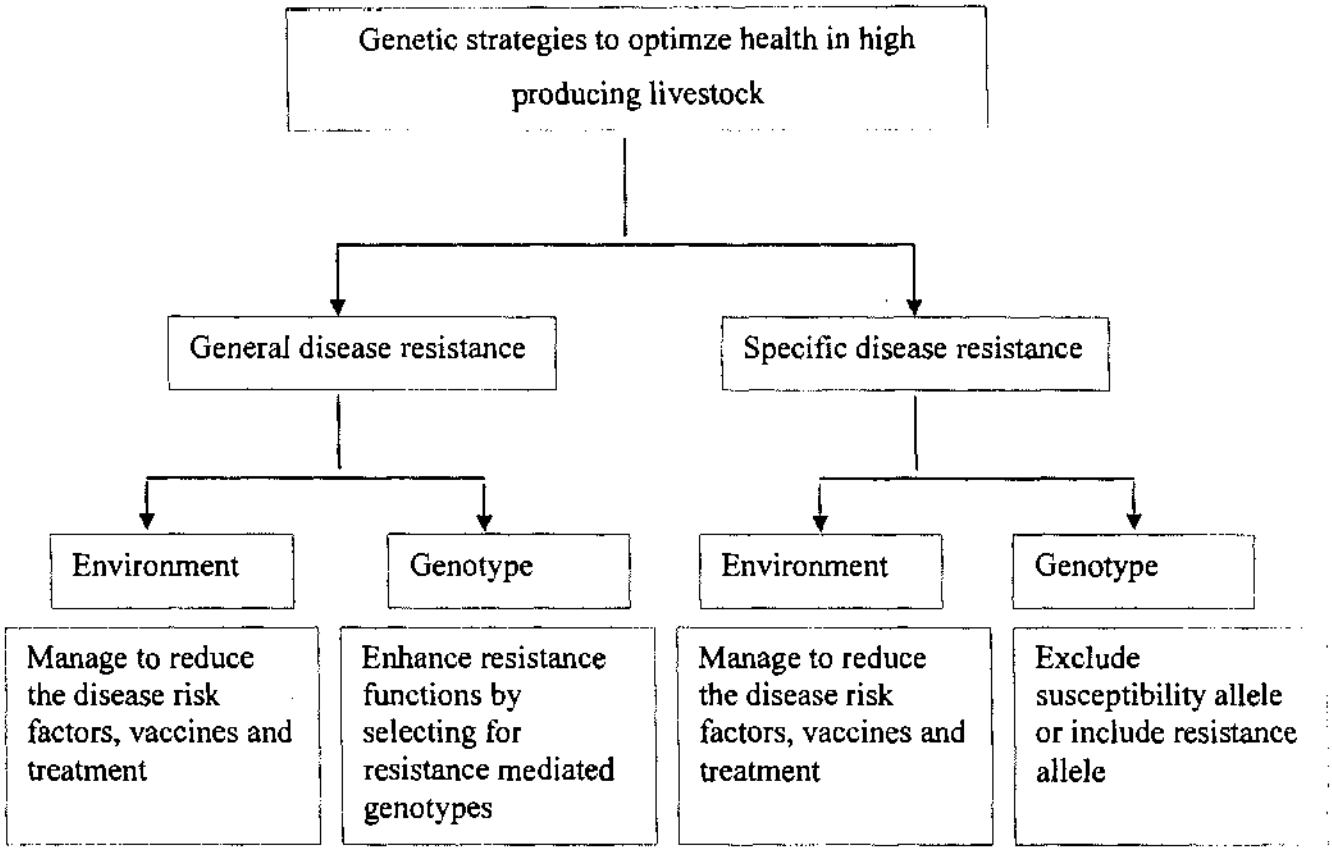


Fig. 2. Genetic strategies for enhancing health in livestock. (From Willkie and Mallard In: Owen, J.B. and Axford, R.F.E. (eds) Breeding for Disease Resistance in Farm Animals. CAB International, Wallingford)

2.3.1 Traditional method of selection for immune response traits in pigs

The accuracy of traditional genetic selection schemes is dependent on heritability (h^2) of the trait in question as well as the source and quantity of phenotypic information (candidate individual, parents, siblings or progeny). The h^2 indicates the relationship between additive genetic variance and the phenotypic variance and, together with selection intensity and generation interval, dictates the genetic improvement that may

be anticipated. Generally, h^2 for disease resistance is low, hence genetic progress based on selection would be expected to be slow. Heritability of respiratory disease in Landrace and Yorkshire pigs was estimated, from sire components of variance and covariance analysis, as 0.14 within the Swedish pig progeny testing programme, in which incidence of all types of pneumonia diagnosed post-mortem was 22.7 %. Genetic correlations between respiratory diseases and production traits were not significant but sick pigs grew more slowly, were leaner and had smaller area of the longissimus dorsi muscles. Heritability for enteric disease was estimated to be 0.59 in a similar study (Lundheim, 1988). Resistance to porcine brucellosis was shown to be heritable and controlled by relatively few genes (Cameron *et al.*, 1940, 1942). Mating of resistant x resistant phenotypes produced 128 progeny of which 76.6% were resistant, 22.6% were of uncertain status and 0.8% were susceptible after one generation of mass selection. Resistance to intracellular bacterial infection, including brucellosis, in mice, cattle and other species has been attributed to a gene, *Nramp*1, within the *Ity/Lsh/Bcg* locus (Qureshi *et al.*, 1996).

2.3.2 Susceptibility and resistance controlled by single alleles

Disease caused by defective alleles can be resolved in populations by testing for the phenotype of the allele itself and reducing the frequency of the unfavourable allele by culling or by avoiding use of carriers in breeding. The Porcine stress syndrome (PSS) phenotype depends upon inheritance of an allele *(ryr*1) of the skeletal muscle ryanodine receptor, a calcium release channel, which has a mutation involving C to T substitution at base pair 1843 (Saiki *et al.*, 1991). The mutation predisposes to stress-induced malignant hyperthermia (MHS) due to abnormal release of Ca^{2+} from the sarcoplasmic reticulum of skeletal muscle, possibly as a result of altered control of Mg^{2+} in the myoplasm (Owen *et al.*, 1997). Pigs that are susceptible to PSS and MHS are lean and have relatively low fat content in muscle and adipose tissue. The *ryr1* mutation is associated with halothane-induced tremors and halothane challenge identifies PSS, however, detection of the *ryr1* mutation at the genetic level more accurately identifies homozygous or heterozygous pigs for implementation of control procedures (Rempel *et al.*, 1993) and has been used successfully in the control of PSS.

Deficiency of the complement regulatory protein, factor H, in Norwegian Yorkshire pigs is inherited as an autosomal recessive trait with complete penetrance and is associated with early piglet death due to membranoproliferative glomerulonephritis induced by deposition of complement component C3 (Jansen *et al.,* 1995). Plasma factor H measurements by enzyme-linked immunosorbent assay (ELISA) identify homozygous deficient and heterozygous healthy carriers which represented 13.5 % of the population (Hogasen *et al.,* 1997). To eradicate the disease, carriers were excluded from breeding.

Several other pig diseases have been described that involve defective alleles, or are suggestive of allelic control of physiological functions or susceptibility to neoplasia. Inherited vitamin D-deficiency rickets occurs in homozygotes that are deficient in renal 25-hydroxycholecalciferol-1-hydroxylase (Winkler *et al.,* 1986). The bleeding disorder, porcine von Willebrand disease, is associated with a restriction fragment length polymorphism in or near the locus controlling the von Willebrand factor (vWF) and likely represents a mutation in the vWF gene (Bahou *et al.,* 1988). Mutations associated with the apolipoprotein B gene are associated with hypercholesterolaemia and coronary artery disease due to atherosclerosis (Rapacz *et al.,* 1986; Maeda *et al.,* 1988). Heritable myasthenia and tremor of leg muscles in Pietran pigs (Campus Syndrome) (Richter *et al.,* 1995) and hypertrophic cardiac myopathy occurring at a frequency of 5.26% in Durocs, 22.98% in Landrace and 5.56% in Yorkshires in Taiwan (Huang *et al.,* 1996) have also been described, but the genetic basis for these is not known. Cutaneous malignant melanoma of Sinclair miniature pigs is inherited and controlled by two loci, one within the major histocompatability gene complex (MHC), which produces a phenotype in which a mutant allele of the second non-MHC locus can initiate tumour development (Tissot *et al.,* 1987). An inherited form of lymphosarcoma has been described in Large White pigs (McTaggart *et al.,*1982). Although the prevalence of these conditions is low in commercial pigs, they may be useful models of human diseases and illustrate the potential for unfavourable mutant alleles to arise in pigs and to cause economically important disease.

Intestinal brush border cell receptors for the three known variants (K88ac, K88ab and K88ad) of the K88 fimbrial adhesin of enteropathogenic *Escherichia coli* are expressed in susceptible pigs (Edfors-Lilja, 1991). In a sample of 24 pigs in each of the Chester White, Duroc, Hampshire and Yorkshire breeds, overall frequency of the receptor-negative and hence resistant phenotype, was 28 %, although breeds differed in receptor phenotype (Baker *et al.,* 1997). Inheritance of the K88ac non-receptor phenotype was thought to be recessive, although this is not yet confirmed (Edfors-Lilja, 1991). Similarly, binding of K99-positive enteropathogenic *E. coli* to pig intestine requires expression of a ganglioside receptor, the active form of which is most prevalent in piglets, the susceptible age group, rather than adults (Teneberg *et al.,* 1990). Receptors for the F18 fimbriated *E. coli* that cause post-weaning diarrhoea and oedema disease of pigs are the dominantly expressed *B* allele in susceptible pigs at the ECF18R locus while resistant animals carry the recessive b allele (Vogeli *et al.,* 1996).

2.3.3 Susceptibility and resistance controlled by multiple alleles

For the resistance against many complex diseases, inheritance is polygenic and controlled by multiple quantitative trait loci (QTL) which may be occupied by alleles favourable or unfavourable to the trait. The phenotype is due to the sum of favourable and unfavourable alleles (Soller, 1994). Identification of QTLs is facilitated by progress in genome mapping. However, QTL mapping requires comprehensive genome maps and a large number of within-family phenotyped individuals, identifying these for each of the many target infectious diseases is a challenge (Soller, 1994). If favourable phenotypes can be quantified and heritability is sufficiently high, it may be possible to enhance resistance by selective breeding (Gavora and Spencer, 1983; Mallard *et al.,* 1992, 1998; Wilkie *et al.,* 1998). Variation in phenotype of resistance-mediating functions is controlled by QTLs, the additive effect of which results in the observed phenotype. The greatest influence upon variation in QTLs may come from regulatory genes rather than from structural genes and, in immune system performance, the cytokine genes may have an important influence (Mitchison, 1997). The polarized antibody and cell-mediated immune (CMI) responses of inbred mouse strains, which are associated with resistance and susceptibility to extracellular and

intracellular pathogens respectively, are controlled by cytokines produced in characteristic patterns, Th1 and Th2, which steer the immune response towards CMI or antibody production respectively (Reed and Scott, 1993; Wilkie *et al.*, 1998). Early in infection, interaction between pathogens and cells of the innate resistance-mediating system, such as natural killer (NK) cells, can induce cytokines that direct development of a response that is characteristic of the host parasite interaction and leads to resistance or susceptibility as a function of the host genotype in inbred mice (Reed and Scott, 1993).

The pig MHC, or SLA, has been tested for influence on many traits with varying results. Correlations between SLA haplotypes or alleles and immune response have been reported. In Large White pigs immunized with bovine immunoglobulin, dinitrophenyl, human serum albumin, sheep red blood cells, poly-L-lysine, hog cholera virus, polydextran or HEWL, only HEWL induced responses correlated with the SLA haplotypes studied, and the correlations were most obvious at low immunizing doses (Vaiman *et al.*, 1978). Both SLA class I and II alleles differed in frequency by social rank in offspring of Yorkshire, Dutch Landrace pigs and the distribution also correlated with varying response to pseudorabies virus infection (Hessing *et al.*, 1994) and to both antibody and CMI (Hessing *et al.*, 1995). Miniature pigs bred for fixed SLA haplotypes (Sachs *et al.*, 1976) have been used to estimate SLA effects on several traits. One of the miniature pig haplotypes, *SLA a/a* was associated with higher response by individuals of several breeds to *B. bronchiseptica* bacterin (Rothschild *et al.*, 1984). *The SLA a/a* haplotype was also associated with significant reduction in muscle larvae of *Trichinella spiralis* after primary infection (Madden *et al.*, 1990). In contrast, the haplotypes *SLA d/d* , *SLA d/g* and *SLA g/g* which share MHC class II genes, were better than *SLA a/a* , *SLA a/c* , *SLA a/d* , *SLA c/c* and *SLA c/d* in producing antibody to HEWL, sheep red blood cells and (T,G-A--L) and in developing DTH to PPD (Mallard *et al.*, 1989a). In this study, frequency of non-responders to immunization was not influenced by SLA haplotype, and while SLA effects were related to differences in immune response much greater effects were associated with dam, sire and litter. Similarly, the *SLA d/d* , *SLA d/g* and *SLA g/g* haplotypes had significantly more serum IgG than the others (Mallard *et al.*, 1989b) and *SLA d/d* pigs

produced antibody of greater avidity (Appleyard *et al.,* 1992b). The SLA haplotypes did not differ significantly in lytic complement activity (CH50), however, the *SLA d/d* , *SLA d/g* and *SLA g/g* haplotypes did not increase CH50 after vaccination. Significant differences in serum CH50 were observed in comparisons between two other SLA haplotypes (Vaiman *et al.,* 1978b). In response to vaccination with an *aro*A mutant *Salmonella typhimurium*, litter had a significant effect on antibody response to the O-polysaccharide (O-ps) but SLA haplotypes varied significantly in lymphocyte blastogenesis induced by O-ps, in that *SLA d/d* , *SLA d/g* and *SLA g/g* behaved as a response group having lower early and higher late responses than the other haplotypes (Lumsden *et al.,* 1993). Haplotype had a significant effect on *in vitro* uptake and killing of *Staphylococcus aureus* and *S. typhimurium*, again with *SLA d/g* and *SLA g/g* acting as a response group (Lacey *et al.,* 1989). Classically, the MHC gene products behave as response/non-response regulators in inbred strains (Benacerraf and McDevitt, 1972), while associations reported for pigs do not suggest control of non-response but rather of the quantity and quality of immune response. This may reflect contribution of linked genes for complement (Lie *et al.,* 1987), tumour necrosis factor, the transporters of antigenic peptides (TAP1 and TAP2) (Vaske *et al.,* 1994) and the low molecular weight polypeptide complex (LMP) that proteolytically processes endogenous antigen for binding to MHC I.

3 Materials and methods

3.1 Materials

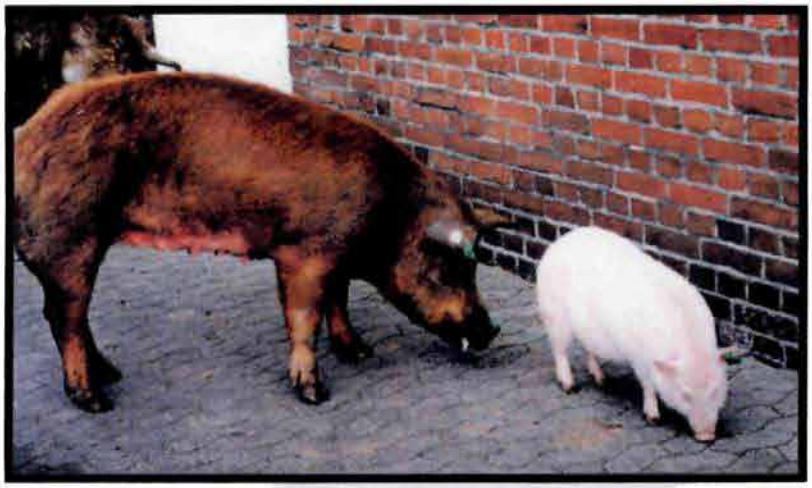
3.1.1 Experimental animals

For this study, one individual for each of the pig breeds, Duroc, German Landrace, Hampshire, Pietrain and F1 cross of Duroc and Berlin minature were used for screening polymorphism in porcine *IL 12p40*, *IL 12p35* and *C5* genes. To determine genetic variation in porcine *IL 12-p40*, *IL 12p35* and *C5* genes, the DUMI resource population pigs (Fig. 3), three commercial pig breeds (German Landrace, Large White and Pietrain) and Indian native breed of pigs were used. Immunological parameters measured in F2 generation of DUMI population were used for the association study with genetic markers of porcine *IL 12p40*, *IL 12p35* and *C5* genes.

3.1.1.1 Resource population

Resource population for this study was generated based on a reciprocal cross of Duroc and Berlin miniature pig breeds (Hardge *et al.*, 1999). Five sows of Berlin miniature pigs were crossed with a Duroc boar and four Duroc sows were crossed with a Berlin miniature boar by AI to produce F1-animals (parental generation). About 47 F1 animals were generated and were crossed between them to produce F2 animals of Berlin-Bonn-DUMI resource population. In this study, 472 F2-Bonn-DUMI animals were used to investigate genetic variation in porcine *C5* gene. The F2-Bonn-DUMI-animals were generated from 11 sows and 3 boars of F1-generation and were reared in the research farm of Frankenforst, Institute of Animal Breeding Science, University of Bonn. These F2-Bonn-DUMI animals were used to investigate complement activity and immune responsiveness, the data of which was used to carry out association studies.

(a)



(b)

(c)

Fig. 3. The Berlin-Bonn-DUMI resource population is generated based on a reciprocal cross of Duroc (left) and Berlin miniature pig (right) grandparent gerneration-F0 (a), parent generation-F1 (b), and F2-generation showed phenotypic segregation (c)

3.1.2 Biological materials, reagents, kits and buffers

The biological mateirals, reagents, kits and buffers used in this study are given below

3.1.2.1 Chemicals, biological materials and kit

Applied Biosystems: Universal PCR master mix for TaqMan allelic discrimination assay, SNP Genotyping assay

Biomol (Hamburg): Phenol, Lambda DNA *Eco*91I (BstE II) and Lambda DNA *Hind*III

Biozym Diagnostik (Hessisch-Oldendorf): Sequagel XR sequencing gel (National Diagnostics) and SequiTherm Excel™II DNA sequencing kit (Epicentre Technologies)

Clontech (Heidelberg): SMART™ RACE cDNA Amplification Kit

Qiagen (Hilden): RNeasy Mini Kit (RNA purification kit)

Gibco/BRL, Life Technologies (Karlsruhe): Trizol™ reagent and SuperScript™II reverse transcriptase

MWG Biotech (Ebersberg) and Sigma (Germany): Oligonucleotide primers

New England Biolabs: Restriction enzymes Bsm I, Aat II, Dde I, Nla IV with their respecitive 10 x buffers.

Pharmacia (Freiburg) and Sigma (Germany): *Taq* polymerase

Promega (Mannheim): DTT, pGEM®-T vector, Pfu DNA polymerase and 10x reaction buffer, Restriction endonucleases *Rsa I,* 10 x BSA and 10 x buffer, Rnase free-Dnase, Rnasin Ribo-nuclease inhibitor, RQ1 Rnase-free Dnase and 10 x buffer, T4 DNA ligase and 2x rapid ligation buffer.

Roche Diagnostics GmbH (Mannheim): Expand high fidelity PCR system

Roth (Karlsruhe): Acetic acid, Agar-Agar, Ampicilin, Ammonium peroxodisulpahte (APS), Boric acid, Bromophenol blue, Chlorofrom, Dimethyl sulfoxide (DMSO), dNTP, EDTA, Ethanol, Ethidium bromide, Formaldehyde, Formamide, Glycerin, Hydrochloric acid, Hydrogen peroxide (30%), IPTG, N,N´-dimethylformamide, Nitric acid, Peptone, Proteinase K, SDS, Silver

nitrate, Sodium carbonate, Sodium chloride, Sodium hydroxide, TEMED, Tris, X-gal, Xylene cyanol, Yeast-extract, and petridish plate (Ø = 9 mm.)

SERVA Electrophoresis GmbH (Heidelberg): Acrylamide molecular biology grade and Bisarylamide.

Sigma-Aldrich Chemie GmbH (Taufkirchen): 2,2′Azino bis (3-ethylbenzthiazo-line-6-sulphonic acid) diammonium salt (ABTS), Agarose, Blue dextran, Calcium chloride, Diethyl barbituric acid, EGTA, Isopropanol, Magnium chloride and Taq polymerase.

Stratagen (Amsterdam): 5α DH *Escherichia coli* competent cell.

The vectorette system was obtained from Sigma – Genosys.

3.1.2.2 Buffers and Reagents

All solutions used in this investigation were prepared with deionized millipore water and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

Acrylamide (40%)	Acrylamide	0095.0 g
	Bis-acrylamide	0005.0 g
	Water (dis.) added to	0250.0 ml
Dextran blue buffer:	Dextran blue (50mg/ml)	0001.0 ml
	EDTA (0.5 M)	0050.0 µl
	Formamide	0005.0 ml
IPTG solution	IPTG	0001.2 g
	Water added to	0010.0 ml
LB-agar plate:	Sodium chloride	008.0 g
	Peptone	008.0 g
	Yeast extract	004.0 g
	Agar-Agar	012.0 g
	Sodium hydroxide, 1N	480.0 µl
	Water added to	800.0 ml

LB-broth:	Sodium chloride	008.0 g
	Peptone	008.0 g
	Yeast extract	004.0 g
	Sodium hydroxide, 1N	480.0 µl
	Water added to	800.0 ml
10 x FA buffer:	MOPS	0041.8 g
(pH 7.0)	Sodium acetate	0004.1 g
	EDTA (0.5M)	0020.0 ml
	Water added to	1000.0 ml
FAA-gel (1.2 %)	Agarose	0001.2 g
	10 x FA buffer	0001.0 µl
	Ethidium bromide	0001.0 µl
	Formaldehyde (37%)	20 µl
	Water added to	100 ml
PBS	Sodium chloride	8766.0 mg
	di-Sodium hydrogen phosphate	1495.0 mg
	Potassium dihydrogen phosphate	0204.0 mg
	Water added to	1000.0 ml
Siver staining solution	Sodium carbonate	0030.0 g
	Formaldehyde	0650.0 µl
	Water added to	1000.0 ml
SSCP loading buffer	Formamide	(95 %)
	Sodium hydroxide	(10 mM)

	Bromophenol blue	(0.25 %)
	Xylene cyanol	(0.25%)
TAE (50x) buffer, pH8	Tris	0242.0 mg0
	Acetic acid	0057.1 ml
	EDTA (0.5 M)	0100.0 ml00
	Water added to	1000.0 ml
TBE (10x) buffer:	Tris	0108.0 g
	Boric acid	0055.0 g
	EDTA (0.5 M)	0040.0 ml
	Water added to	1000.0 ml
TE buffer:	Tris (1 M)	0010.0 ml
	EDTA, (0.5 M)	0002.0 ml
	Water added to	1000.0 ml
X-gal	X-gal	0050.0 mg
	N,N´-dimethylformamide	0001.0 mll

3.1.2.3 Software Programs

BLAST program	http://www.ncbi.nlm.nih.gov/blast/
Multiple Sequence Alignment	http://saturn.med.nyu.edu/searching/promultali.html http://prodes.toulouse.inra.fr/multalin/multalin.html
Restriction enzyme analysis	http://www.firstmarket.com/cutter/cut2.html http://tools.neb.com/NEBcutter/index.php3
Image Analysis program (version 4.10)	Li-cor Biotechnology

Primer design	Primer Express -ABI prism
	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
One-D scan program	Scanalytics Inc., Billerica, MA
SAS version 8.0	SAS Institute Inc., Cary, NC
Pig Genome	http://linkage.rockefeller.edu/soft/list.html
	http://www.marc.usda.gov/genome/genome.html
	http://www.genome.kvl.dk/piggenome/misc.html
	http://www.genome.iastate.edu/pig
	http://nitro.biosci.arizona.edu/zbook/book.html
Radiation Hybrid Panel	http://imprh.toulouse.inra.fr/
DNA to protein translate tool	http://us.expasy.org/tools/dna.html

3.1.2.4 Equipments

Automated sequencer (LI-COR 4200)	MWG Biotech, Ebersberg, Germany
Centrifuge	Hermle, Wehingen, Germany
Carbon dioxide incubator	Heraeus, Hanau, Germany
Electrophoresis (for agarose gels)	BioRad, München, Germany
Electrophoresis (vertical apparatus)	Consort, Turnhout, Germany
PCR thermocycler (PTC100)	MJ Research, USA & BioRad, Germany
Power Supply PAC 3000	BioRad, München. Germany
Spectrophotometer UV/visible light (DU-62)	Beckman
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany
Real time PCR	ABI-Applied biosystems, USA

2 Methods

In this charpter, the basic molecular genetics methods used in this study are descriped first, followed by the methodology used for sequence identification and SNP screening for the three genes (*IL 12p40*, *IL 12p35* and *C5*) are given. The overview of the present study is given in fig. 4.

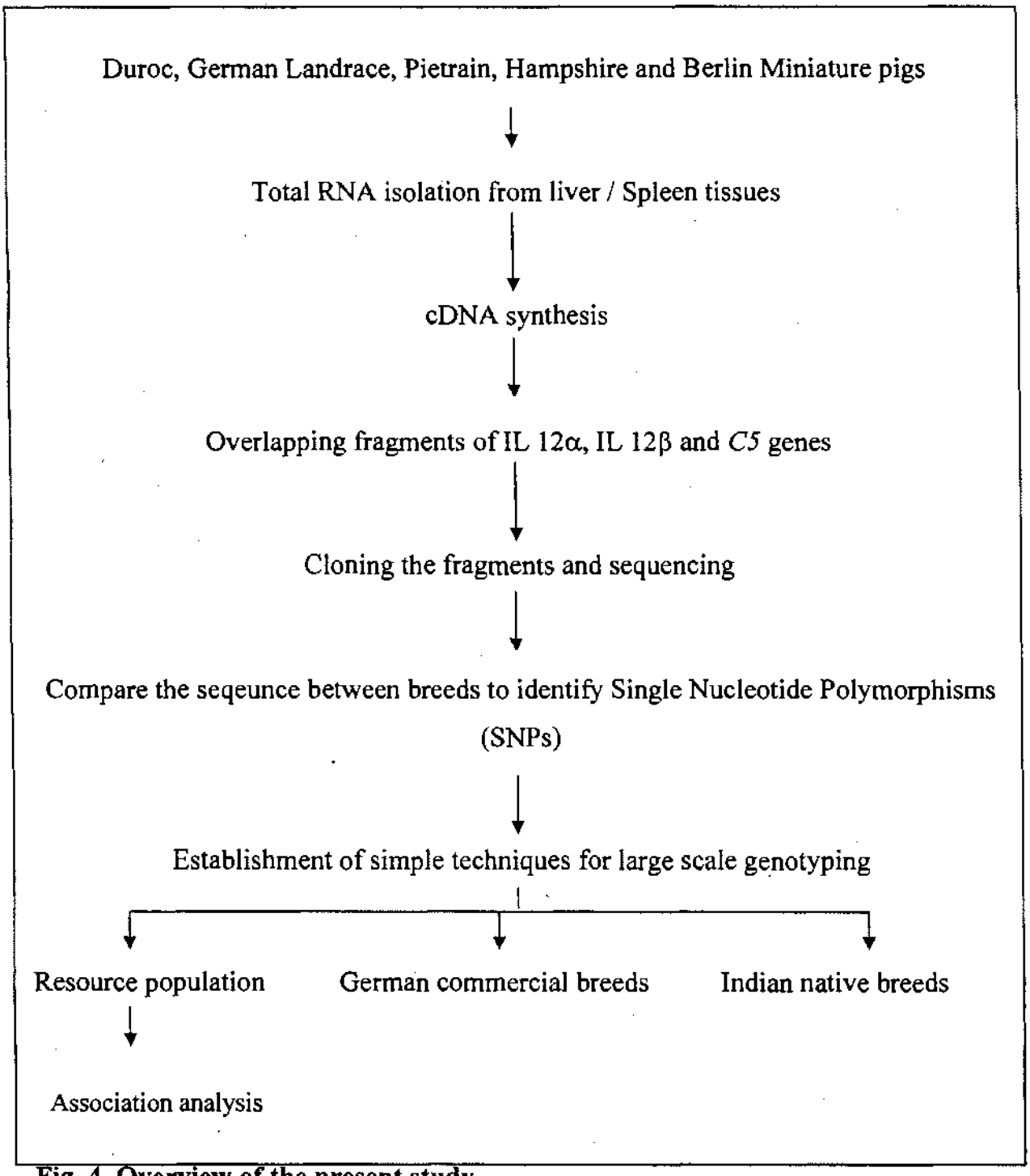


Fig. 4. Overview of the present study

3.2.1 Molecular genetic methods

3.2.1.1 RNA isolation and cDNA synthesis

RNA isolation

Total RNA was isolated from liver and spleen tissues from one animal each of Duroc, Hampshire, Pietrain, German Landrace and F2 animal of DUMI population. The tissue samples were stored at –80 °C and were taken out just before isolation. About 5 to 10 mg of tissue was homogenized by using mortar and pestle and incubated with 1ml of TRIZOL for 5 minutes at 15 to 30 °C to permit complete dissociation of nucleoprotein complexes. Then 0.2 ml of chloroform was added, mixed thoroughly and incubated at 15 to 30 °C for 2 to 3 minutes. The samples were centrifuged at 12,000 g for 15 minutes at 2 to 8 °C. Following centrifugation, the mixture separates into a lower red phenol-chloroform phase, a white interphase and a colorless upper aqueous phase. RNA remains exclusively in the upper aqueous phase. The aqueous phase was transferred to a fresh tube and the RNA was precipitated by adding 0.5 ml of isopropyl alcohol. Then the samples were incubated at room temperature (15 to 30 °C) for 10 minutes and centrifuged at 12,000 g for 10 minutes at 2 to 8 °C. After precipitating, the RNA pellet was washed once with 70 % ethanol. Then the RNA pellet was air dried and dissolved in 20 µl of DEPC treated distilled water.

DNAse digestion and quality checking of RNA

The RNA extracted was digested with DNAse (RNAsin 1 µl, RNA 32 µl, 5 x DNAse digestion buffer 10 µl, DTT 5 µl and DNAse 2 µl) for one hour at 37 °C to digest the DNA residue, if there is any. The digested RNA was purified using RNeasy purification kit (Qiagen) as per the manufacturer's instruction. The quality of RNA was checked by running 2 µl of total RNA in 1.5 % FAA gel. The good quality RNA was used for further analysis. OD spectrometer value was taken to estimate the

concentration of total RNA. One OD_{260} = 40 µg/ ml of RNA. The RNA was stored at –80 °C for further use.

cDNA synthesis

About 2 µg of total RNA was reverse transcribed using reverse transcriptase to synthesis cDNA. Two µg of total RNA and 1 µl of oligo T $dT(11)_N$ (5′-TTTTTTTTTTTN-3′) was added in a 0.2 ml PCR tube and the final volume was made up to 6 µl with DEPC treated water and incubated at 70 °C for 5 minutes and immediately snapped in ice. Then 5 µl of 5 X RT PCR buffer, 5 µl of 10 mM dNTPs, 2.5 µl of 0.1 M DTT, 0.5 µl of RNasin and 1µl of SuperScriptII™ RNase H reverse transcriptase (200 unit/ µl) (Gibco BRL) was added and incubated at 42 °C for 90 minutes followed by 70 °C for 5 minutes. The synthesized cDNA was diluted with 80 µl of DEPC treated millipore water and stored at –20 °C for further use.

Purity checking of cDNA

The isolated cDNA was checked for any DNA contamination by amplifying a portion of β actin, a house keeping gene by PCR. Primers were designed covering an intronic region of the gene and hence when amplified, will produce a high molecular weight fragment from genomic DNA than from cDNA. Amplification of 2 µl of cDNA/genomic DNA was performed with Taq polymerase (Sigma) in a final reaction volume of 20 µl, containing 10 mM each of dNTPs, 50 nanomoles of each of primers (forward: 5′-gagaagctctctgctacgtcga-3′ and reverse: 5′-cagacagcaccgtgttggc-3′) in 1 x PCR buffer in thermal cycler for 35 cycles at 95 °C denaturation, 56 °C annealing and 72 °C extension followed by 5 minutes extension at 72 °C. The products were checked in 2 % agarose gel along with a genomic DNA control. The good quality cDNA should have single low molecular weight fragment than the genomic DNA control.

3.2.1.2. Polymerase Chain Reaction (PCR)

About 400 to 450 bp of gene specific products were amplified by using AccuTaq LA polymerase (Sigma) or Taq polymerase (Sigma) in a 20 µl reaction volume containing 2 µl of cDNA or genomic DNA, 10 mM of each of dNTPs, 50 nanomoles of each of the primers (Tables 1, 2, 3 & 4) in 1 X PCR buffer containing 1.5 mM $MgCl_2$ in a thermal cycler (MJ Research) for 40 cycles at 95 °C denaturation, annealing at 50 to 65 °C (varies for different primer combinations) and 72 °C extension followed by 10 minutes extension at 72 °C.

3.2.1.3 Cloning and sequencing of PCR fragment

DNA isolation from agarose gel

The amplified products were electrophorosed in 0.7 % agarose gel having long combed well. The product was visualized under UV transilluminator and the gel having the DNA fragment of interest was cut and DNA was isolated from gel by phenol-chloroform extraction method. The gel was homogenized in 0.5 ml of 1 x TE buffer, mixed with 0.5 ml of phenol-chloroform and centrifuged at 10,000 RPM for 15 minutes. The supernatant was removed to a fresh tube and 0.5 ml of chloroform was added, mixed thoroughly and centrifuged at 10,000 RPM for 10 minutes and DNA was precipitated with double the volume of chilled absolute alcohol after adding $1/20^{th}$ volume of 3 M Sodium acetate (60 µl) and stored at –20 °C overnight for complete precipitation of DNA. The extracted DNA was washed with 70 % alcohol and dissolved in 5 to 7 µl of distilled water, which was used for further analysis.

A-tailing of blunt ended PCR fragments

The pGEM-T vector (Promega), used in this study for cloning, contains a 'T' overhang to ensure ligation of the products amplified by Taq DNA polymerase as Taq polymerase, during amplification, will add the adenine (A) residue in 3' end. But when

proof reading polymerase like Pfu DNA polymerase or AccuTaq LA polymerase was used, which produce blunt ended fragment, the 3'end should be tailed with adenine (A) residue to ensure ligation. About 1-7 µl of purified PCR fragment generated by a proofreading polymerase was taken in a 0.2 ml PCR tube and 1 µl 10 x reaction buffer with 1.5 mM $MgCl_2$, dATP to a final concentration of 0.2 mM and 0.5 U of Taq Pol. were added and reaction volume was made up to 10 µl with deionized distilled water. The reaction mixture was incubated at 70 °C for 15 to 30 minutes.

Ligation

The ligation reaction was setup in a 0.5 ml PCR tube wherin 2.5 µl of 2 x Rapid ligation buffer, 0.5 µl of pGEM – T vector (50 ng / µl), 0.5 µl of T4 DNA ligase (3 Units / µl) and 1.5 µl of A-tailed Template DNA were added and mixed by pipetting. The reaction was incubated at room temperature (16 – 20 °C) for 3 hours or overnight at 4 °C.

Transformation

The tubes containing the ligation reaction were spinned breifly to collect the contents at the bottom of the tube. About 3.5 µl of each ligation mixture was added to a sterile 15 ml tubes, kept in ice. The competent cells (JM109 Strain or DH5α strain of *E. coli*) for transformation were removed from -80 °C just prior to adding and thawed by placing in ice (about 5 minutes). The cells were mixed by gently flicking the tube and 60 µl of competent cells was transfered into a 15 ml tube and incubated along with 3.5 µl of ligation mix in ice for 30 minutes. Then, the competent cells were heat shocked at 42 °C for 90 seconds in a water-bath and immediately snapped in ice for 2 minutes. Finally, 650 µl of LB broth was added and incubated at 37 °C in shaking incubator for 90 minutes.

Cloning

Two LB/ ampicillin/ IPTG/ X-gal plates for each ligation was prepared by adding 20 µl of 0.1 M IPTG and 20 µl of X-gal (50 mg/ml) to the LB plates with ampicillin and spread with a glass spreader. The solutions were allowed to absorb for 20 minutes prior to use. The transformation culture was plated to LB/ ampicillin/ IPTG/ X-gal plates in duplicate. The plates were incubated at 37 °C overnight until colonies are visible. Successful cloning of an insert in the pGEM-T vectors interrupts the coding sequence of β-galactosidase, hence the vectors with insert produce white color colonies in plate having X-gal medium against blue colony producing vectors which doesn't have inserts. Two white colonies and a blue colony (negative control) are picked in 30 µl 1 x PCR buffer, from each plate. The bacteria were lysed by heating at 95 °C for 15 minutes. The colonies were screened for the insert by performing a PCR with primers designed in M13 promoter region of the vector in 20 µl of reaction volume containing 10 µl of lysate, 10 mM each of dNTPs, 50 nanomoles of each of primers (forward: 5′-ttgtaaaacgacggccagt-3′, reverse: 5′-caggaaacagctatgacc-3′), 0.5 U of Taq polymerase (Sigma) in 1 x PCR buffer were amplified in PTC 100 (MJ Research) thermal cycler for 35 cycles at 95 °C denaturation, 65 °C annealing and 70 °C extension followed by 10 minutes of final extension at 70 °C. The products were electrophoresed in 2 % agarose gel. Clones having insert would be having higher molecular weight fragments than the blue clones.

Sequencing

The positive clones were sequenced by using LI-COR automated DNA sequencer. The sequencing is performed by dye termination method. For each sample, 4 reactions were performed, each reaction will be having one dideoxy nucleotide termination mix (ddATP, ddTTP, ddCTP and ddGTP) which will terminate the elongation reaction once the nucleotide binds to the elongating strand. The primers used were designed from Sp6 (5' taaatccatgtgtatcttatg-3') and T7 (5'-attatgctgctgagtgatatatcccgct-3') promoter regions of the pGEM-T vector and is flourescent labelled which can be laser

read by LI-COR automated DNA sequencer. Four sequencing reactions of the different termination mixes were setup for each PCR fragment. Each reaction consists of 1µl of termination mix and 2 µl of premix solution (3.6 µl of sequencing buffer, 0.8 pM of 700 IRD-labeled Sp6 primer, 0.8 pM of 800 IRD-labeled T7 primer, 1.5 µl of M13 PCR product and 2.5 U Taq polymerase). PCR was performed in a thermal cycler (MJ Rescarch) for 35 cycles with 95 °C denaturation, 59 °C annealing and 72 °C extension, followed by 5 minutes extenison at 72 °C. Immediately after PCR, 1.5 µl of stop buffer was added and the product was stored at –20 °C till loading. Immediately before loading, the sequencing reactions were denatured at 85 °C and loaded onto 41 cm 6 % Sequagel-XR (National Diagnostics, Biozyme). Electrophoresis was performed on a LI-COR model 4200 automated DNA sequencer in 1x TBE buffer at 50 °C, 50 W and 1500 V. Sequence data was analysed by using Image Analysis program, version 4.10 (LI-COR Biotechnology).

3.2.1.4 Comparative sequencing for SNP detection

One sample from each of the 5 breeds (Duroc, Hampshire, German landrace, Pietrain and F1 cross of Duroc and Berlin minature) were sequenced and compared among them to detect polymorphism. Multiple sequence alignment was performed using the software from Internet (http://saturn.med.nyu.edu/searching/promultali.htmlor http://prodes.toulouse.inra.fr/multalin/multalin.html). The SNPs detected were confirmed by sequencing the sample again. Once the SNPs were established PCR based faster and cost effective protocols like PCR-Restriction fragment length polymorphism (PCR-RFLP), PCR-Single strand conformation polymorphism (PCR-SSCP) and TaqMan allelic discrimination assay were employed to facilitate large scale genotyping.

3.2.2 Sequence identification and SNP screening in genes

3.2.2.1 Porcine Interleukin 12-*p40* (*pIL 12p40*) gene

Interleukin 12 (*IL 12*) is a heterodimeric cytokine consisting of glycosylated subunits of 35 and 40 kDa linked by disulfide bonds (Podlaski *et al.*, 1992). These two subunits were produced by two unrelated genes located in two different chromosomes. The genes were amplified in overlapping fragments by using gene specific primers. A 2124 bp cDNA of porcine Interleukin 12 *p40* (*pIL 12p40*) is amplified in overlapping fragments using primers which were designed based on the published sequence (Accession No. U08317). The sequences of the primers are given in table 1. The fragments were amplified, cloned and sequenced by standard protocols. The schematic representation of the amplification of *pIL 12p40* gene is given in fig. 5.

Table 1. List of primers used for amplifying *pIL 12p40* gene.

Primer Name	Forward Primer	Reverse Primer
IL 12-p40-1	tggttgtctcctggttttcc	cgtccggagtaattctttgc
IL 12-p40-2	tgaccatccacgtcaaagag	ttgtgaacagcttccagcac
IL 12-p40-3	cattgaggtcgtgctggaag	aggacacagatgcccattcac
IL 12-p40-4	tgggtgggaacacaagagat	ggctaggcacttctggtcac
IL 12-p40-5	aagacatgatggagcagacc	gtggcactggaattaggaca

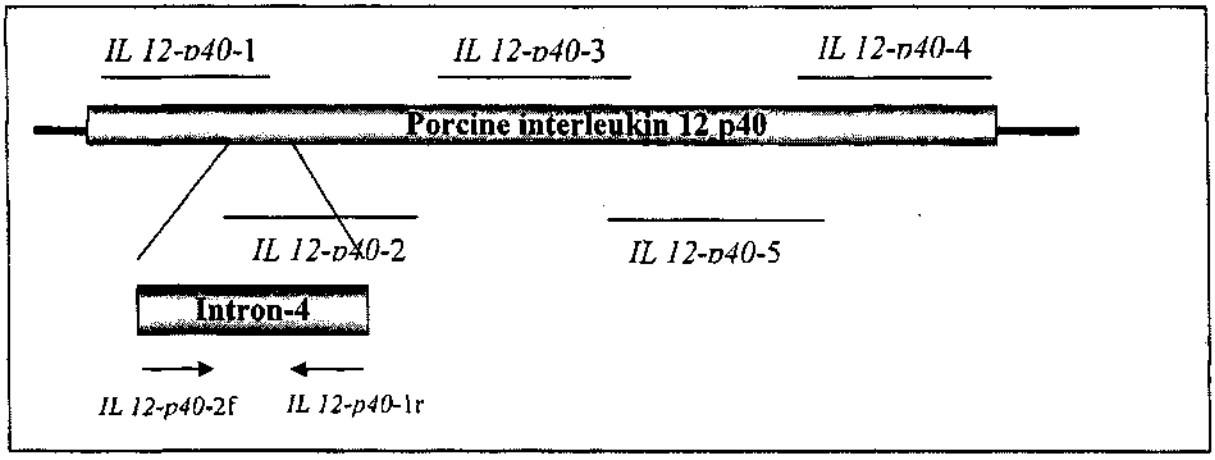


Fig. 5. Schematic display of the 5 overlapping PCR fragments and Intron-4 amplification plan for screening polymorphisms in porcine *IL 12 p40* gene.

3.2.2.2 Porcine interleukin 12-*p35* (*pL 12p35*) gene

Similarly a 1500 bp cDNA of *pIL 12p35* was amplified in overlapping fragments using primers based on the published sequence (Accession No. L35765.). The sequences of the primers are given in table 2. The overlapping fragments were amplified, cloned and sequenced by standard protocols as described above. The schematic representation of the amplification of *pIL 12-p35* gene is given in fig. 6.

Table 2. List of primers used for amplifying *pIL 12p35* gene.

Primer Name	Forward Primer	Reverse Primer
IL 12-p35-1	tggttgtctcctggttttcc	cgtccggagtaattctttgc
IL 12-p35-2	tgaccatccacgtcaaagag	ttgtgaacagcttccagcac
IL 12-p35-3	gagtcatcaccccaatt	caatccacttgggaat
*IL 12-p35*i2	tggttgtctcctggttttcc	catttgaacattcctgggcct
*IL 12-p35*i4	gcacagtggaggcctgcttac	cgtccggagtaattctttgc
*IL 12-p35*i5	tgaccatccacgtcaaagag	tgcattcatggcctggaactc

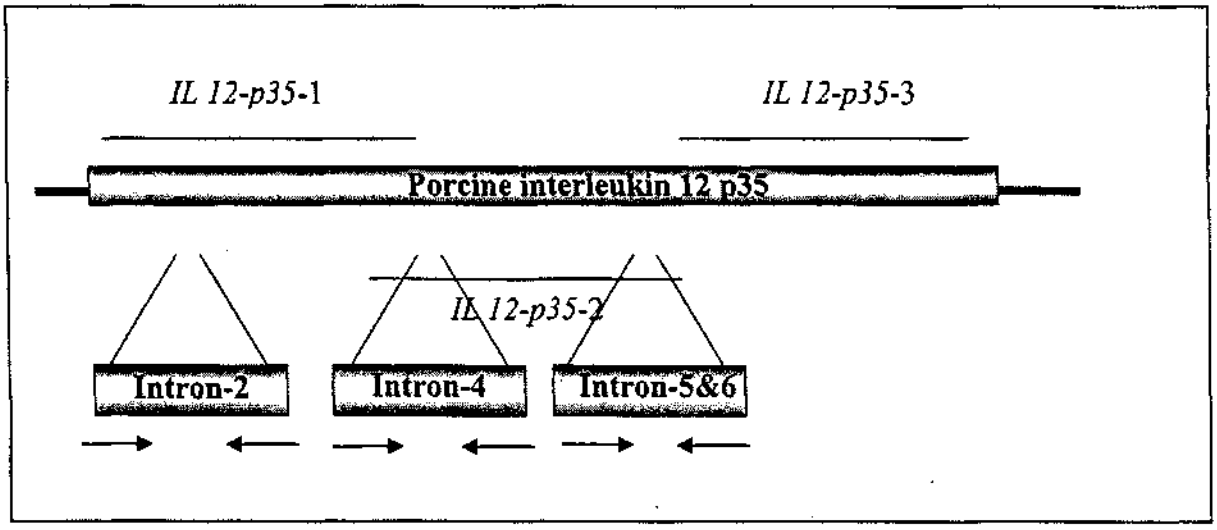


Fig. 6. Schematic display of the 3 overlapping PCR fragments and 4 Introns amplification plan for screening polymorphisms in porcine *IL 12 p35* gene.

3.2.2.3 Identification of promoter region of *pIL 12p35* gene

Vectorette PCR

Vectorette system from Sigma-Genosys was used to identify promoter region of *IL 12p35* gene. Five different vectorette ends viz. Bam HI , Cla I, Eco RI, Hind III and Blunt ends (e.g. Alu I, Eco RV, Hae III, Pvu II, Rsa I, Sma I) were used for screening in this study. Porcine genomic DNA was digested with the above restriction enzymes and ligated with respective vectorette ends. First round of PCR amplification using vectorette system as template was performed with vectorette primer and a gene specific primer (*IL 12p35*i2). Using the first round PCR products as template second round nested PCR was done using nested vectorette primer and nested gene specific primer (*IL 12*-pro: 5'-cacccagtgtctttcccccct'). The products were electerophoresed in 0.7 % agarose gel and DNA isolated from agarose gel. The isolated DNA products were cloned in pGEM-T vector and sequenced by using LI-COR automated sequencer.

3.2.2.4 Porcine complement component *C5*

The porcine complement component 5 (*pC5*) was not yet sequenced. In this study complete *pC5* was sequenced based on the sequence information from 3 ESTs of *pC5* (Acc No. BF713665, D84333 and D84332). Primers were designed based on the conserved sequence region of human and mouse *C5* cDNA sequence (Table 3). Rapid Amplification of cDNA ends – polymerase chain reaction (RACE-PCR) was performed to amplify 5' end of *C5* cDNA.

RACE ready cDNA synthesis

The 5' RACE – Ready cDNA was prepared as per the manufacturers protocol (CLONTECH). Breifly, I-3 µl of liver RNA sample, I µl of 5' CDS primer and 1 µl of SMART II A oligo for 5' RACE – Ready cDNA were taken in a 0.2 ml PCR tubes and final volume was made upto 5 µl with DEPC treated sterile water. The contents

were mixed and incubated at 70 °C for 2 min and immediately snaped in ice for 2 min. Then 2 µl of 5x First strand buffer, 1 µl of DTT (20 mM) and 1 µl of dNTP mix (10 mM) and 1 µl of powerscript reverse transcriptase were added and contents were gently mixed. Then the tubes were incubated at 42 °C for 1.5 hr and finally the RACE ready cDNA was diluted with 100 µl of Tricine EDTA buffer. The tubes were heated at 70 °C for 10 min and the samples were stored in –20 °C for further use.

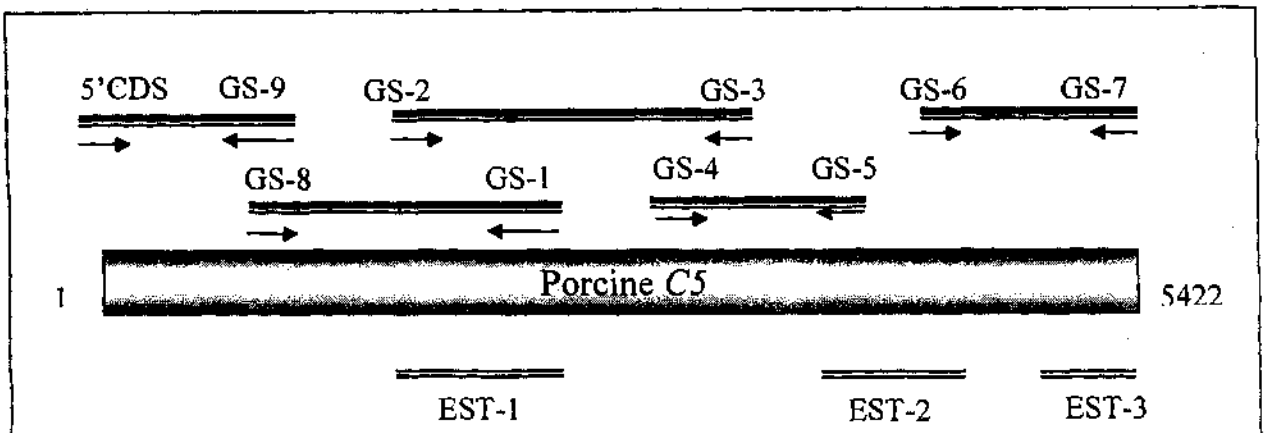
Rapid Amplification of cDNA ends (RACE)

The gene specific primers for *pC5* were designed based on the sequence information obtained from three ESTs. (BF713665, D84333 and D84332.). RACE ready cDNA were used as template for *C5* amplification. PCR was performed with 5' RACE ready cDNA with 5' CDS primer and one gene specific primer at the 5' end. The 3' end of cDNA was amplified by using gene specific primers designed based on the conserved sequence region of human and mouse cDNA. The primer sequences for this analysis are given in table 3 and the complete amplification plan is given in fig 7.

Table 3. Primer sequences for the sequence identification of porcine *C5* gene.

Primer Name	Forward Primer
RACE	ctaatacgactcactatagggcaagcagtggtatcaacgcagagt
GS-1	cagcccaggtcattcttctc
GS-2	cagctccaggttcatctgtc
GS-3	ccgcctctgcactcccttt
GS-4	tgacgacttgaagccagcca
GS-5	actgtagccaagccactgttc
GS-6	ggtgcctctcaatgatgacc
GS-7	gctattcttcagtccctggc
GS-8	gaagccagccaaaagagaa
GS-9	ctgatccttggggatctatg

After identification of complete sequence of *C5*, primers were designed to amplify overlapping fragments of 400 to 500 bp from 5 different breeds of pigs to identify single nucleotide polymorphisms (SNPs). The primers used to amplify the overlapping fragments are given in table 4.



- 5' CDS – Primer specific for 5' RACE – Ready cDNA
- GS-1, GS-2, GS-5, GS-6 & GS-7 – Gene specific primer designed based on sequence information known
- GS-3, GS-4 GS-8 & GS-9 – Gene specific primer designed based on the conserved region of human and mouse *C5* cDNA sequence
- EST –1, 2 and 3: Expressed sequence Tags, submitted to Genbank with accession numbers BF713665, D84333 and D84332, respecitvely.

Sequence information already known

Fig. 7. Amplification plan of complement component *C5* cDNA.

Table 4. List of primers used for amplifying *C5* gene for polymorphism screening.

Primer Name	Forward Primer	Reverse Primer
C5-01	ggcctttggggaatactttg	ctgatccttggggatctatg
C5-02	gaagccagccaaaagagaa	caacattgtgttccgcattg
C5-03	aatgcggaacacaatgttga	aaactccagcaccgtcactc
C5-04	caggcaagagcaaattaccg	agccacccaggaatccaac
C5-05	acgataggctccatcataacagg	cagctccaggttcatctgtc
C5-06	cgggaaaatgatgaaccttg	gcacctttgcattgagggta
C5-07	cctgattccctaaccacctg	tttgacaccttctgcaccac
C5-08	cgagtggtgccagaaggtgtc	agggggcacagatcaaaag
C5-09	gtgccccctgatgaaaaatc	acctctactggcctcccaat
C5-10	ggtgcctctcaatgatgacc	ggttacatgctgtttctttc
C5-11	cacagaccagataaacagtg	tgtaggccaatattcaatcc
C5-12	gtacattacccttagatcc	gctattcttcagtccctggc

The overlapping fragments were amplified, cloned and sequenced by standard protocols as described above. The schematic representation of the amplification of p*C5* gene is given in fig. 8.

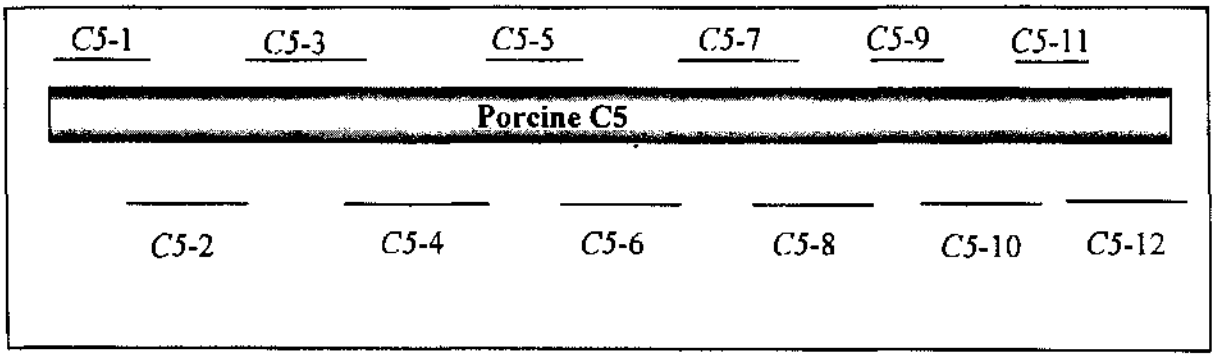


Fig. 8. Schematic display of the overlapping PCR fragments amplification plan for screening polymorphisms in porcine *C5* gene.

3.2.3 Genotyping and chromosomal localization of gene

Simple protocols like PCR-RFLP, PCR-SSCP, TaqMan allelic discrimination assay using Real-time PCR were established for the SNPs detected to facilitate large scale genotyping. Radiation-Hybrid mapping panel and linkage mapping was used to localize the genes to various chromosomes of pigs. The procedures are breifly given below.

3.2.3.1 Polymerase Chain Reaction – Restriction fragment length polymorphism (PCR-RFLP)

Once mutations were established, genomic DNA covering the polymorphic region was amplified with specific primers. Restriction digestion of the product was carried out in a 25 µl reaction volume containing 1 to 10 U of specific Restriction Enzyme (Table 5) and 10 µl of PCR products in 1 X RE buffer and incubated at 37 °C for 4 to 6 hours. The digested products were seperated in 2 - 3% agarose gel.

Table 5. Details of SNPs genotyped by PCR-RFLP.

Name of the gene	SNP position	Primer - Forward	Primer – Reverse	Restricition Enzyme
IL 12p40	193rd of Int-IV	tgaccatccacgtcaaagag	cgtccggagtaattctttgc	Nla IV
C5	2766th of *C5*	cgtggagggaatctgttcat	tttgacaccttctgcaccactc	Dde I
C5	3018th of *C5*	ggagaggtcatgtctgcagtt	tgcaggttctgtctttgagc	Bsm I §
C5	1044th of *C5*	caggtggattttctgaagagg	ggaatcccaggtttcacaaa	Rsa I
C5	1203rd of *C5*	gcaggttaaggaactcgcttg	aaactccagcaccgtcactc	Aat II

§ - The restriction enzyme Bsm I was digested at 65 °C

3.2.3.2 Polymerase chain reaction – Single strand conformation polymorphism (PCR-SSCP)

PCR-SSCP was performed to genotype the polymorphism at position 447 of intron-4 of *IL 12-p40* gene. The genomic DNA (200 to 300 bp), covering the polymorphic region, was amplified using gene specific primers (Fw - cgtccggagtaattctttgc and Re - gggctgacagccttgaaa). The amplified fragments were mixed in the ratio of 1:10 with SSCP loading buffer (95 % formamide, 10 mM NaOH, 0.25 % bromophenol blue, 0.25 % xylene cyanol), denatured at 95 °C for 5 min and immediately snapped in ice. The products were electrophoresed in 12 % polyacrylamide gel (49:1 acrylamide/ bisacrylamide) at 4 °C for 5 hours, at I2 W in 0.5 X TBE. The SSCP fragments were visualized by silver staining. Breifly, the gels was fixed with acetic acid solution for 20 to 30 minutes followed by in Nitric acid solution for 20 to 30 minutes. Then the gel was washed twice with millipore water and impregnated with silvernitrate by keeping in silver nitrate solution for 30 minutes. Bands were developed by using sodium carbonate solution having formaldehyde and after bands were developed, fixed by acetic acid. The gels were dried in gel drier for 2 hours at 70 °C and documented.

3.2.3.3 TaqMan allelic discrimination using real-time PCR

Primers and probe for genotyping polymorphism in the promoter region of IL 12p35 gene were synthesised by 'Assay by design service of SNP genotyping' of Applied Biosystems. The primer and probe sequences are

Primers: Fw- cgtgtcaccgagaagctgat and Re - ggactccagtctcttgctttcttt

Probes: VIC - CTGTGTCTCGCTCTAC and FAM

TCTGTGTCTCTCTCTAC

The primers and probes were synthesised as 80 x concentration.

The reaction mix (6.25 µl of TaqMan Universal PCR master mix, 5.59 µl fo DNAse free distilled water and 0.16 µl of 80 X assay mix) was prepared and added to 96 well plate having 0.5 µl genomic DNA of 2 positve control for each of the allele, samples to be genotyped and 2 controls without template. Plates were 'preread' in ABI prism 7000 instrument and PCR was performed (initial denaturation of 95 °C for 10 minutes and 40 cycles of 92 °C for 15 sec and 60 °C for 1 min). After PCR amplification endpoint plate read was performed. The SDS software calculates the fluorescence measurements made during the plate read and plots Rn values based on the signals from each well. Using the software the SNPs were determined for each sample.

The process for analyzing data for SNP assays involves the following procedures:

1. Creating and setting up an allelic discrimination plate read document
2. Performing an allelic discrimination plate read on an SDS instrument
3. Analyzing the plate read document
4. Making manual allele calls
5. Confirming allele types

3.2.4 Chromosomal localization of gene

3.2.4.1 Mapping using Radiation Hybrid Panel

The genes were mapped using Radiation Hybrid mapping panel of INRA, France. PCR was carried out for each gene, with gene specific primers (Table 6) that amplifies genomic DNA for 118 pig-hamster hybrid clones along with a pig and a hamster DNA as a positive control. The PCR products were run on 2 % agarose gel and the data for

all the 118 clones (1 or 0 for presence or absence of band) were submitted to IMpRH webserver of INRA, France (http://imprh.toulouse.inra.fr/) to find the relative position of the gene to the markers already mapped.

Table 6. Primer sequences used for mapping using RH mapping panel.

Name of the gene	Primer - Forward	Primer – Reverse
pIL 12p40	Fw: tgaccatccacgtcaaagag	Re: cgtccggagtaattctttgc
pIL 12p35	Fw: gaggcacgacaggactttcc	Re: cccggagttaatccgaaagc
pC5	Fw: cagctccaggtcatctgtc	Re: cagcccaggtcattcttctc

3.2.4.2 Linkage Mapping

The data obtained from genotyping Bonn DUMI resource population was used for linkage mapping of the genes. The data was checked for any genotyping errors by using Pedcheck (Version 1.1). Two point linkage analyses was carried out by using CRI-MAP package (Version 2.0). The results obtained were also used to confirm the results obtained by RH mapping panel.

3.3 Statistical analysis

Analyses of variance were performed with the procedure "Mixed" and 'repeated' statement of the SAS software package (SAS System for Windows, Release 8.02) to investigate effects on hemolytic complement activity (alternative and classical) and serum complement C3 protein concentration. General linear model was applied to find the effects of genotypes for antibody titre against Mycoplasma, Aujesky and PRRS vaccines. Model was used essentially as given below in order to identify other significant environmental and genetic effects apart from the *IL 12* and *C5* genotype and its interaction by stepwise elimination of non-significant effects. Those factors found to significantly affect the phenotypes were included in a model to assess association of *IL 12 & C5* with the parameters of complement activity. Animal effect was the subject specified in the repeated statement in case of complement activity traits. Four covariance structures, autoregressive (AR), heterogenous autoregressive (ARH), compound symmetry (CS) and hetrogenous compound symmetry (CSH) were

investigated to fit a specified covariance structure with the data of each traits. Of each immunological traits that were mentioned above, the least squares means between *IL 12 & C5* genotype classes on each time of blood sampling were compared by PDIFF function of SAS and was corrected for the unequal class number by using Tukey-Krammer correction.

The model given below is for complement activity traits

$Y_{ijklmnop} = \mu + sire_i + dam_j + parity_k + treatment_l + genotype_m + time_n + sex_o + litter\ size_p + animal_{ijklmnop} + genotype*time + \varepsilon_{ijklmnop}$

where:

y_{ijklmn}: traits: CH_{50}, AH_{50},

μ: overall mean

$sire_i$: fixed effect of sire; i=1-3

dam_j: random effect of dam

$parity_k$: fixed effect of parity; k=1-5

$treatment_l$: fixed effect of treatment vaccinated/non-vaccinated control

$genotype_m$: fixed effect of genoptype; m=1-2 (for *IL 12-p40*) 1-2 (for *IL 12p35*) 1, 2 & 3 (for *C5*)

$time_n$: fixed effect of time point of measurement prior and after vaccinations; m=1-8

sex_o : fixed effect of sex; o =1, 2

genotype*time: intereaction effect between genotype and time of blood sampling

'animal'$_{ijklmnop}$: random effect of animal

$\varepsilon_{ijklmnop}$: residual error

The model for antibody titres against mycoplasma, aujeszky and PRRS is given below

$Y_{ijklmn} = \mu + sire_i + dam_j + parity_k + genotype_l + sex_m + litter\ size_n + animal_{ijklm} + \varepsilon_{ijklmn}$

where:

y_{ijklmn}: traits: Antibody titre values before and after vaccination of mycoplasma and aujeszky and after vacination of PRRS.

μ: overall mean

$sire_i$: fixed effect of sire; i=1-3

dam_j: random effect of dam

$parity_k$: fixed effect of parity; k=1-5

$genotype_l$: fixed effect of genoptype; l=1-2 (for *IL 12-p40*) 1-2 (for *IL 12p35*) 1, 2 & 3 (for *C5*)

sex_m : fixed effect of \sex; m=1,2

litter $size_n$: fixed effect of litter size n=1-4

$'animal'_{ijklm,}$: random effect of animal

ε_{ijklmn}: residual error

4 Results

4.1 Interleukin 12

Interleukin 12 is a heterodimeric cytokine consisting of glycosylated subunits of 35 and 40 kDa linked by disulfide bonds. These 2 subunits are secreted by 2 different genes (*IL 12p40* and *IL 12p35*) located in 2 different chromosome. The mRNA sequence of the both the genes in pigs has already been published (Foss and Marutaugh, 1997).

4.1.1 Porcine interleukin 12 *p40* (*pIL 12 p40*)

A 2124 bp cDNA of *pIL 12p40* gene has already been sequenced by Foss and Maurtaugh, 1997 (Accession No. U08317). It contains an open reading frame of 324 amino acids. The predicted porcine amino acid sequence has a four amino acid deletion relative to that of human *IL 12p40* and is 84 % and 70 % homologous to the predicted human and murine amino acid sequences, respecitively. Potential N- linked glycosylation sites and cysteine residues are conserved between all three species. The 3' untranslated region contains 12 AUUUA repeats (Foss and Murtaugh, 1997).

pIL 12p40 contains 7 exons similar to that of humans. In this study intron-4 of the gene was amplifed, cloned and sequenced. It is about 561 bp long which is comparable with that of human sequence (542 bp) in length. The sequence is given in fig. 9.

Exon3...TAATTCCATTCCCTTGGATGGTGTCAATTTTCTCTTTTGCTCCAAAAGAGATTGAA
AAAAATAAAAATTTAAAAGGCAAGACATAATTATGTAGTTAAAGAAATAATATCAAGTC
TCCTACTGGAACCACCTGAAATCTACATTTTCCTTTGCTTTGTTTTTCCTTTGCTGGACACT
TTGGGTTATTAGCCATCAAAATCTCATGTTGAGTTCATGGGGATTGCATGCTTTTCCTGCT
GTTCTTTTTAAATAACAGTGACATTACTGGATGGCGAATGGAAGTTAACGTGCATAGTGC
ATAACTCAGCACTGGGCTGACAGCCTTGAAAAAACTTAATACCTCCTTTCGATATGTAGT
ATACTCTATAGAATCAGCATCCTGTTGRGACAGTCCCaGTCTTTGCTGAACCACCTTCAAG
GGGAACAGCAGCATTTTCCAAGGRAATTTATGGATTATCTTCGCTATTCAGTGGCCTGTG
ATTCATCGCAATAAAAATCTTCATAATTTTaAAAGARAGAKAMCAAACCCCTAAACACTG
AATTTTTGTTCTTTTCAAATCC...Exon4

Fig. 9. Sequence of Intron-4 of *IL 12p40* gene.

4.1.1.1 Polymorphism Screening

Based on the above sequence information of porcine *IL 12p40*, five primer pairs were designed to amplify overlapping PCR fragments of about 400-500 bp from spleen cDNA of one animal from each of the five breeds viz. Duroc, German Landrace, Hampshire, Pietrain, and F1 cross of Duroc x Berlin Miniature Pig. The amplified products were run on 0.7 % agarose gel and the band was excised and DNA was isoloated. All of the PCR fragments were cloned, sequenced and compared to identify polymorphisms. No mutations could be detected between the breeds in cDNA sequences. However, some variations could be detected between the sequence from genbank and the present sequence. Since no mutation could be detected in exonic region of the gene, introns were screened for single nucleotide polymorphisms (SNPs). A 542 bp of Intron 4 of *pIL 12p40* gene was amplifed by using primers of the flanking region. The sequence comparision showed 2 mutations between the breeds. One was at position 196 of Intron 4, a transition mutation from A to G (Fig.10) and the other was at position 447 which was a transition mutation from T to C Fig.10).

The first polymorphic site at position 196 could be detected by using PCR-SSCP (Fig 10). The second polymorphic site at position 447 could be detected by PCR-RFLP as the Restriction enzyme Nla IV cuts the DNA of the individuals having C variants as compared to T variants (Fig.10). The SNPs were found to be seggregating in the DUMI resource population. These two SNPs were found to be segregating in 3 genotypic patterns (AC/AC, AC/GT and GT/GT) in DUMI resource population. These SNPs were also genotyped in German commercial breeds and Indian native pigs. The gene and genotype frequencies of the SNPs for commercial breeds are given in table 7.

Table 7. Gene and Genotype frequencies of *pIL 12p40* alleles.

Breeds	*pIL 12p40* Int-IV-196					*pIL 12p40* Int IV-447				
	AA	AG	GG	A	G	TT	TC	CC	T	C
German Landrace	0.23	0.48	0.29	0.47	0.53	0.19	0.50	0.31	0.44	0.56
Pietrain	0.12	0.26	0.62	0.25	0.75	0.05	0.24	0.71	0.17	0.83
Large white	0.10	0.26	0.64	0.23	0.77	0.06	0.35	0.59	0.235	0.765
Indian native	-	-	-	-	-	0.07	0.53	0.40	0.33	0.67

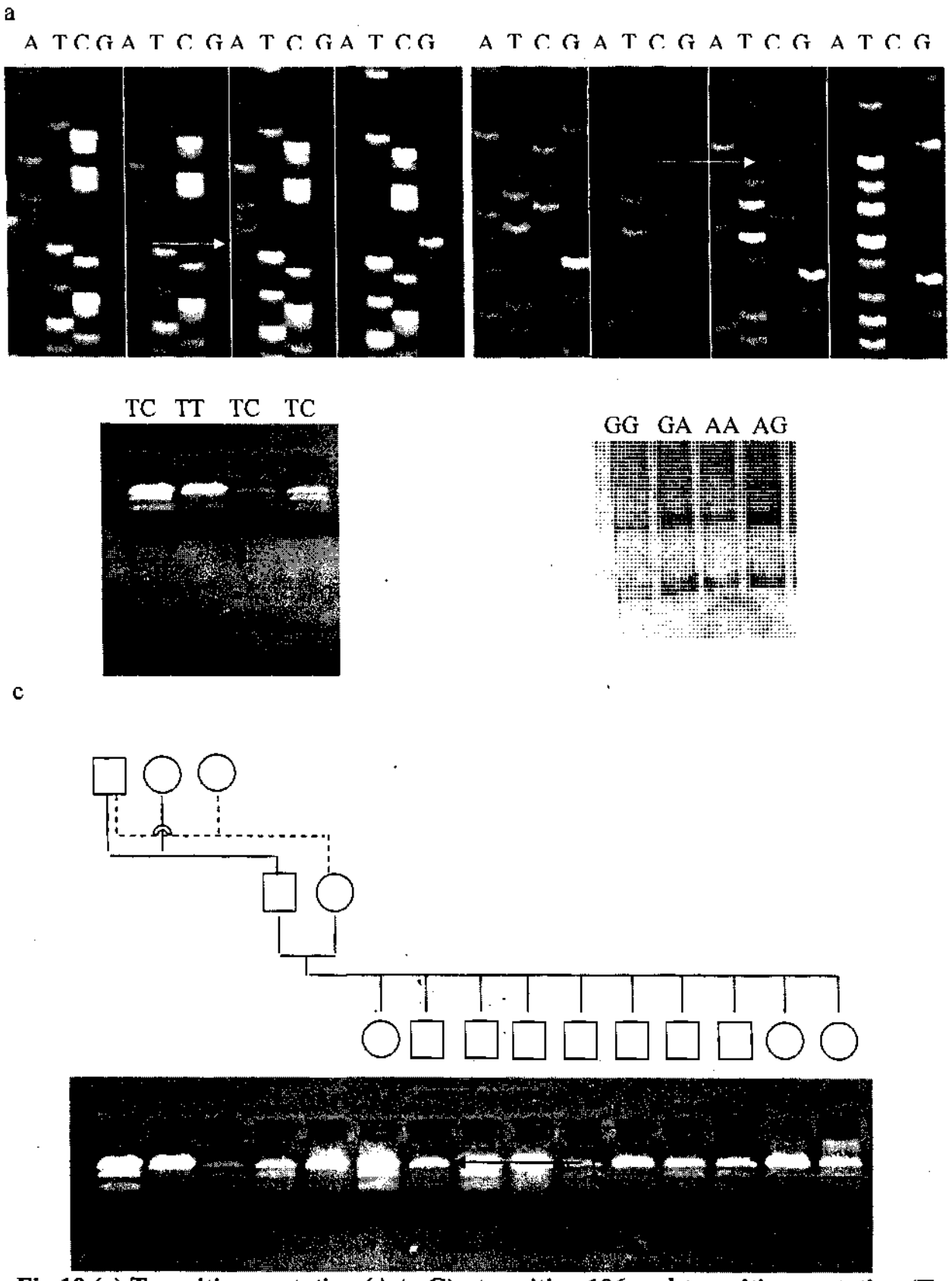


Fig 10 (a) Transition mutation (A to G) at position 196 and transition mutation (T to C) at position 437 (b) The two mutations could be detected by PCR-RFLP and PCR-SSCP (c) the mutation at postion 196 was found to be segregating in the resource population, arrows indicating different genotypes.

4.1.1.2 Chromosomal localization of *pIL 12p40*

Mapping using Radiation Hybrid Panel

The *pIL 12p40* was mapped using Radiation Hybrid mapping panel of INRA, France. PCR was carried out by using primers *IL 12-p40*-2Fw: tgaccatccacgtcaaagag and *IL 12-p40*-1Re: cgtccggagtaattctttgc to amplify genomic DNA for 118 pig-hamster hybrid clones along with a pig and a hamster DNA as a positive control. The PCR products were run on 2 % agarose gel and the data for all the 118 clones (1 or 0 for presence or absence of band) were submitted to IMpRH webserver of INRA, France (http://imprh.toulouse.inra.fr/) to find the relative position of the gene to the markers already mapped. The most significantly mapped marker was found to be SW 262 (40.28 cR and LOD = 10.59) which was assigned to chromosome 16 (Rohrer *et al.*, 1996).

Linkage Mapping

The data obtained from genotyping Bonn DUMI resource population was used for linkage mapping of the genes. The data was checked for any genotyping errors by using Pedcheck (Version 1.1). Two point linkage analyses was carried out by using CRI-MAP package (Version 2.0). The results revealed linkage to loci S0111, S0026 and S0061 with recombination fraction of 0.45, 0.29 and 0.30, respectively, on sex averaged map (fig. 11). S0111 has been assigned to Sscr16q11-q12 (Lopez-Corrales *et al.*, 1999).

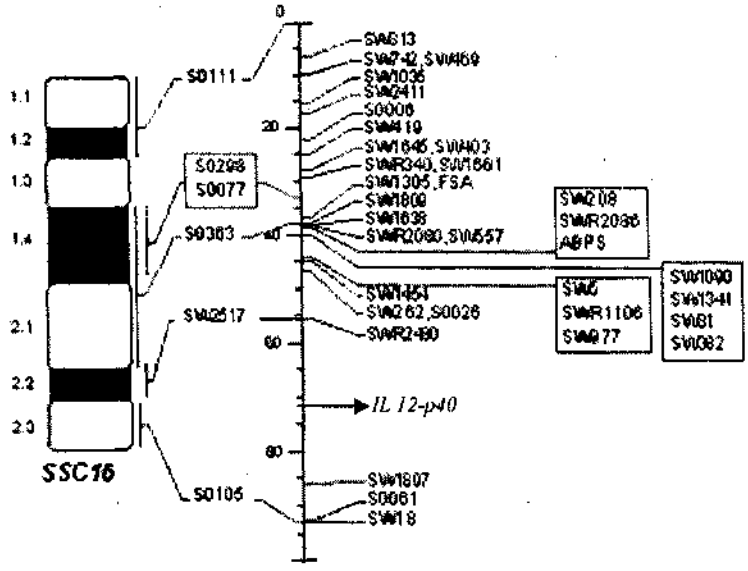


Fig. 11. *pIL 12p40* gene was found to be located in SSC16.

4.1.1.3 Association analysis of *pIL 12p40* genotypes with various immunologial traits in DUMI resource population

Association between *pIL 12p40* markers and various immunological traits was carried out in 417 animals of the DUMI resource population. These animals were immunized with Mycoplasma, Aujeszky and PRRS vaccines. The response of the alternative complement activity, classical complement activity, plasma complement C3 protein concentration, antibody titre against Mycoplasma, Aujeszky and PRRS vaccines, before (time 1. 4 and 7) and after vaccinations (time 2, 3, 5, 6 and 8) were measured, the data of which is used for carrying out association analysis. The values of different traits are given in tables 8, 9, 10 & 11.

The two polymorphic sites of *pIL 12p40* were found to be segregating in the resource population. These two SNPs were found to be segregating in 3 genotypic patterns (AC/AC- *IL 12p40*1/IL 12p40*1*, AC/GT- *IL 12p40*1/IL 12p40*2* and GT/GT- *IL 12p40*2/IL 12p40*2*) in DUMI resource population. No recombination between these 2 markers could be found in the resource population. The number of homozygotes for *IL 12p40*2/IL 12p40*2* genotype was very small, varies between 7 and 12 for different complement traits and hence not considered for the statistical analysis.

Table 8. ACH50 values of alternative complement activity (mean ± SD, minimum and maximum) in porcine serum before and after Mycoplasma, Aujeszky and PRRS vaccinations.

Vaccination	Blood sampling	Number	Mean±SD	Minimum	Maximum
Mycoplasma	Time 1	300	53.64±28.31	11.23	160.57
	Time 2	319	53.22±28.43	10.53	135.45
	Time 3	322	55.05±24.86	10.79	135.76
Aujeszky	Time 4	338	56.86±30.13	10.53	160.61
	Time 5	331	62.31±32.08	10.16	195.04
	Time 6	322	68.46±47.63	10.94	263.65
PRRS	Time 7	315	69.75±41.70	10.05	263.34
	Time 8	330	69.71±37.42	10.67	288.19

Table 9. CH50 values of clasical complement activity (mean ± SD, minimum and maximum) in porcine serum before and after Mycoplasma, Aujeszky and PRRS vaccinations.

Vaccination	Blood sampling	Number	Mean±SD	Minimum	Maximum
Mycoplasma	Time 1	268	40.70±23.13	10.16	170.36
	Time 2	392	48.23±28.81	10.01	215.71
	Time 3	319	47.97±29.19	10.63	232.04
Aujeszky	Time 4	345	59.08±36.46	10.26	278.26
	Time 5	363	64.91±35.71	12.20	294.02
	Time 6	368	59.79±39.10	10.00	297.78
PRRS	Time 7	331	62.53±35.58	10.12	285.10
	Time 8	343	60.99±40.89	10.18	271.76

Table 10. Serum complement component C3c (mean ± SD, minimum and maximum) in porcine serum before and after Mycoplasma, Aujeszky and PRRS vaccinations.

Vaccination	Blood sampling	Number	Mean±SD	Minimum	Maximum
Mycoplasma	Time 1	349	0.162±0.076	0.012	0.417
	Time 2	347	0.169±0.064	0.023	0.417
	Time 3	135	0.115±0.038	0.023	0.194
Aujeszky	Time 4	376	0.193±0.087	0.023	0.444
	Time 5	376	0.198±0.088	0.040	0.500
	Time 6	183	0.124±0.056	0.044	0.690
PRRS	Time 7	177	0.118±0.038	0.047	0.225
	Time 8	176	0.124±0.036	0.044	0.252

Table 11. Antibody titres against Mycoplasma, Aujeszky and PRRS vaccines (mean ± SD, minimum and maximum) of porcine serum before and after vaccinations.

Vaccination	Blood sampling	Number	Mean±SD	Minimum	Maximum
Mycoplasma	Time 1	103	0.44±0.22	0.03	1.38
	Time 3	103	0.76±0.31	0.02	1.54
Aujeszky	Time 4	280	0.42±0.19	0.03	1.38
	Time 6	280	0.64±0.30	0.09	1.54
PRRS	Time 8	087	0.70±0.49	0.03	2.37

Association between *pIL 12p40* markers and complement activity traits

The statistical analysis for various complement activity traits, C3c protein level and haptoglobin values was performed using the repeated statement in the proc mixed procedure of the SAS program. Four covariance structures of Proc mixed repeated statement viz. autoregressive (AR), heterogeneous autoregressive (ARH), compound symmetry (CS) and heterogeneous compound symmetry (CSH) were fitted for each of the trait and the best fit was used in the model. Different factors like dam, sire, litter size, parity, and sex were considered along with genotype and time of vaccination and the factors which was found to be significant was used further in the model. The correlation coefficients between two measures on the same animal at all times are 0.26 and 0.57 for the alternative and classical complement activity traits, respectively. But in case of serum complement C3 protein, correlation was found to be decreasing with increasing length of time interval measures, which ranged from 0.7 between first two measurements to 0.08 between the last two measurements.

Table 12. Effect of *pIL 12p40* marker with various complement activity traits (ACH50 and CH50) and serum complement C3 protein).

Source	Immunological traits			
	ACH50	CH50	C3c	HP
pIL 12p40	**	**	ns	ns
Time	*	*	*	ns
pIL 12p40 *time	ns	**	**	ns

** P<0.01 * P<0.05 ns – Not significance

The statistical analysis revealed a high level of significance between *pIL 12p40* markers for alternative (ACH50) and classical (CH50) complement activities and at different time intervals. Though no significance could be found between the 2 genotypes for serum C3c level, there was a significant effect of time of vaccination as well as time and genotype interaction. No significance between the genotypes as well as between different time intervals could be found for haptoglobin values.

Association between *pIL 12p40* markers and alternative complement activity (ACH50)

The association between *pIL 12p40* markers and alternative complement activity traits at different sampling time is given in table 13. The overall mean of ACH50 of *IL 12p40*1/IL 12p40*1* animals was 4.86 units/ml less than that of *IL 12p40*1/IL 12p40*2* animals, which was found to be statistically significant ($p<0.01$). Moreover, the effect of *IL 12p40* genotypes was found to be significantly different at before and after vaccination of Mycoplasma (time1, time 2 and time 3), whereas no significant difference between the genotypes could be found before and after vaccination of Aujeszky (time 4, time 5 and time 6). In case of PRRS no signifcant difference was found between the genotypes after vaccination though signifcant differnce exits before vaccination.

Table 13. Least squares means with standard errors for alternative complement activity of each *pIL 12p40* genotype at individual sampling times.

Vaccination	Blood sampling	*pIL 12p40* genotype	
		*IL 12p40*1/IL 12p40*1*	*IL 12p40*1 /IL 12p40*2*
Mycoplasma	Time 1	52.89 ± 5.25*	57.14 ± 4.95
	Time 2	52.02 ± 5.21*	62.38 ± 4.78
	Time 3	53.33 ± 5.14*	60.73 ± 4.80
Aujeszky	Time 4	59.62 ± 5.08	60.05 ± 4.74
	Time 5	63.36 ± 5.07	69.39 ± 4.77
	Time 6	75.40 ± 5.12	76.26 ± 4.77
PRRS	Time 7	73.22 ± 5.19*	78.01 ± 4.75
	Time 8	71.14 ± 5.07	75.89 ± 4.72
	Overall	62.62 ± 4.30*	67.48 ± 3.77

* - $p<0.05$

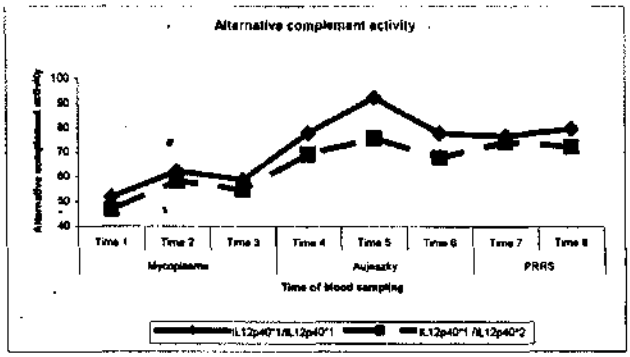


Fig. 12. Graphical representation of alternative complement activity at different time of blood sampling for the 2 *pIL 12p40* genotypes.

Association between *pIL 12p40* markers and classical complement activity (CH50)

The association between *pIL 12p40* markers and alternative complement activity traits at different sampling time is given in table 14. The overall mean of CH50 of *IL 12p40*1/IL 12p40*1* animals was 7.26 units/ml higher than that of *IL 12p40*1/IL 12p40*2* animals, which was found to be statistically significant ($p<0.05$). Moreover, the effect of *pIL 12p40* genotypes was not found to be significantly different at before and after vaccination of Mycoplasma (time1, time 2 and time 3), whereas significant difference in CH50 levels between the genotypes could be found before and after vaccination of Aujeszky (time 4, time 5 and time 6). In case of PRRS, no signifcant difference was found between the genotypes before and after vaccination (time 7 and time 8).

Table 14. Least squares means with standard errors for classical complement activity of each *IL 12p40* genotype at individual sampling times.

Vaccination	Blood sampling	*pIL 12p40* genotype	
		*IL 12p40*1/IL 12p40*1*	*IL 12p40*1 /IL 12p40*2*
Mycoplasma	Time 1	52.08 ± 5.79	47.01 ± 5.23
	Time 2	62.29 ± 5.62	58.43 ± 5.12
	Time 3	58.93 ± 5.48	54.92 ± 5.06
Aujeszky	Time 4	78.09 ± 5.36*	69.46 ± 5.00
	Time 5	92.50 ± 5.36*	75.82 ± 4.96
	Time 6	77.98 ± 5.40*	67.94 ± 4.96
PRRS	Time 7	76.82 ± 5.43	74.38 ± 4.96
	Time 8	79.68 ± 5.41	72.36 ± 4.96
	Overall	72.30 ± 4.92*	65.04 ± 4.35

* - $p<0.05$

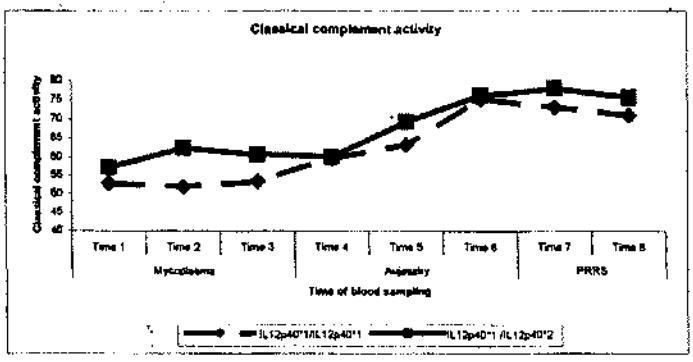


Fig. 13. Graphical representation of classical complement activity at different time of blood sampling for the 2 *pIL 12p40* genotypes.

Association between *pIL 12p40* markers and serum C3c protein and hepatloprotein level

The effect of *pIL 12p40* markers on serum C3 c protein is given in table 15. The overall mean of C3c of *IL 12p40*1/IL 12p40*1* and *IL 12p40*1/IL 12p40*2* animals was 0.17 units/ml and there was no significant difference between the two genotypes. In case of C3c protein level, there was a significant difference at time 4 (before vaccination of Aujeszky), but no significant diffrence existed between the genotypes at any other time. There is a slight increase in values immediately after vaccination except for heterozygotes for aujeszky vaccination.

Table 15. Least squares means with standard errors for C3c protein level of each *IL 12p40* genotype at individual sampling times.

Vaccination	Blood sampling	*IL 12p40* genotype	
		*IL 12p40*1/IL 12p40*1*	*IL 12p40*1 /IL 12p40*2*
Mycoplasma	Time 1	0.15 ± 0.006	0.16 ± 0.006
	Time 2	0.16 ± 0.007	0.16 ± 0.006
	Time 3	0.17 ± 0.008	0.18 ± 0.01
Aujeszky	Time 4	0.18 ± 0.006*	0.20 ± 0.006*
	Time 5	0.19 ± 0.006	0.19 ± 0.006
	Time 6	0.19 ± 0.009	0.18 ± 0.008
PRRS	Time 7	0.17 ± 0.009	0.17 ± 0.009
	Time 8	0.17 ± 0.009	0.17 ± 0.009
	Overall	0.17 ± 0.006	0.17 ± 0.005

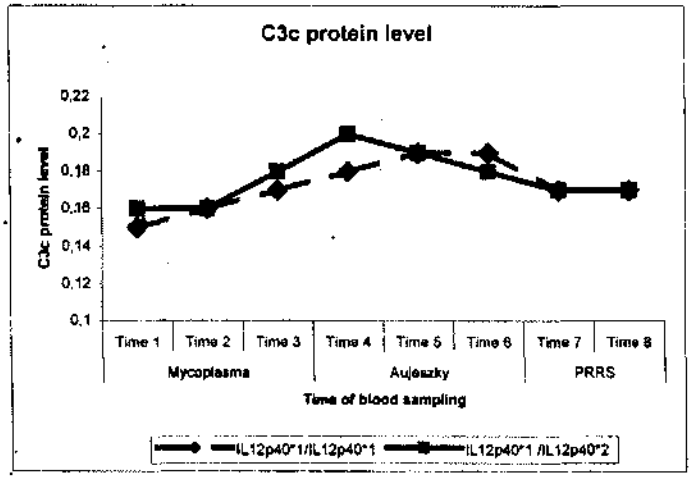


Fig. 14. Graphical representation of C3c protein level at different time of blood sampling for the 2 *pIL 12p40* genotypes.

Association between *pIL 12p40* markers and haptoglobin protein level

The effect of *pIL 12p40* markers on haptoglobin protein levels is given in table 16. The *IL 12p40* genotypes have HP levels of 0.46 and 0.48. The *IL 12p40*1/IL 12p40*1* animals has 0.2 units/ml of HP levels higher than that of *IL 12p40*1/IL 12p40*2* amimals, which was not statistically significant. In case of haptoglobin significant difference exists at time 1 (before vaccination of Mycoplasma) but no significant difference could be found between the genotypes at any other times. The HP level showed a significant increase in its level immediately after vaccination which returns to normal as time progress. There was also increase in level as age increases.

Table 16. Least squares means with standard errors for haptoglobin level of each *IL 12p40* genotype at individual sampling times.

Vaccination	Blood sampling	*IL 12p40* genotype	
		*IL 12p40*1/IL 12p40*1*	*IL 12p40*1 /IL 12p40*2*
Mycoplasma	Time 1	0.28 ± 0.04*	0.48 ± 0.04*
	Time 2	0.46 ± 0.03	0.33 ± 0.04
	Time 3	0.37 ± 0.04	0.37 ± 0.04
Aujeszky	Time 4	0.31 ± 0.04	0.29 ± 0.04
	Time 5	0.76 ± 0.04	0.78 ± 0.04
	Time 6	0.55 ± 0.04	0.56 ± 0.04
PRRS	Time 7	0.43 ± 0.04	0.49 ± 0.04
	Time 8	0.48 ± 0.04	0.49± 0.04
	Overall	0.46 ± 0.02	0.48 ± 0.02

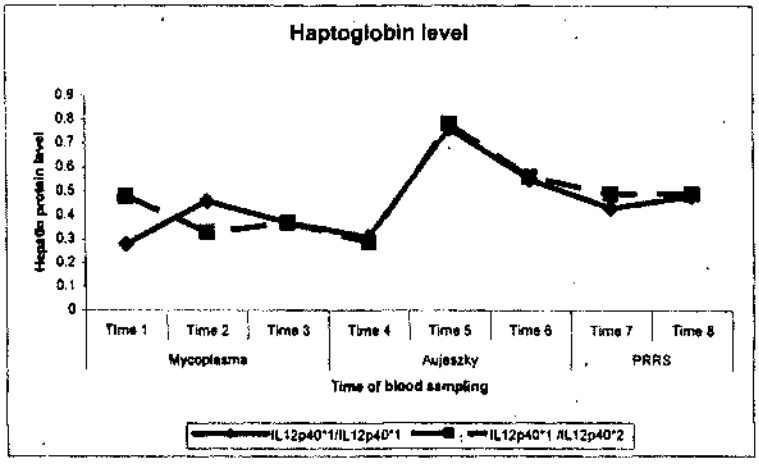


Fig. 15. Graphical representation of haptoglobin level at different time of blood sampling for the 2 *pIL 12p40* genotypes.

Association of *pIL 12p40* markers with various antibody titres before and after vaccination

Association between *pIL 12p40* markers with various antibody titres (Mycoplasma, Aujeszky, PRRS) were analysed by using 'general linear model' procedure of SAS software (v8.02). Various effects like sire, dam, litter size, parity, sex, etc. were cosidered for the analysis and those which have significant effect on the trait were used for analysis. The results are showed in table 17. The results showed that there is no significant effect of markers on antibody titres before as well as after vaccination for all the vaccination traits.

Table 17. Effect of *pIL 12p40* marker with various antibody titres before and after vaccination.

Source	Immunological traits				
	Mycoplasma pre vac	Mycoplasma post vac	Aujeszky pre vac	Aujeszky post vac	PRRS post vac
pIL 12p40	ns	ns	ns	ns	ns

ns – No significance

4.1.2 Porcine Interleukin 12*p35* gene (*pIL 12p35*)

A 1471 bp cDNA off *pIL 12p35* gene has been sequenced by Foss and Maurtaugh, 1997. It contains an open reading frame of 256 amino acids. The predicted amino acid sequence of the porcine *IL 12p35* was compared with that of human and murine. Both the human and procine sequences have an additional upstream start codon in frame which would generate a protein with an additional 34 amino acids at the amino terminal end.

These potential additional amino acids are not required for biological activity of recombinant human protein and the region is only 21 % homologous between swine and human. Nonfunctional upstream start codons have beeen identified in about 10 % of mRNA messages. In addition, the downstream translation start site conforms to a Kozak sequence (CANCAUGGC) for optimal initiation wheras the upstream site does not. The predicted porcine amino acid sequence has an additional three amino acids and 86 % homology to human *IL 12p35* and 7 additional amino acids and 66 % homology to murine *IL 12p35*. The first 25 amino acids of the porcine sequence form a typical signal peptide. Potential N-linked glycosylation sites and cysteine residues are conserved between al three species. The 3' untranslated region contains eight AUUUA repeats that are associated with rapid mRNA degradation in a number of genes (Foss and Murtaugh, 1997).

Interleukin 12*p35* gene has 7 exons and 8 introns, similar to that of human *IL 12p35*. In this study, introns 1, 3, 4 and 5 were completely sequenced (Acccession no. AY388981, AY388982, AY388983 and AY388984). The sequences are given in fig 16. The Introns 1, 3, 4 and 5 were 612, 144, 85 and 168 bp, respectively. Intron-4 has a single sequence repeat (T repeat of 21 bp) which was also reported to be presented in cattle and sheep (Schmidt *et al.,* 2000), but unlike in cattle and sheep where variation in repeat number has been reported, no polymorphism could be detected among the pig breeds screened in this study.

The promoter region of the *IL 12p35* has been identified by using Vectorette PCR technique. A 212 bp of 5' promoter region was identified, cloned and sequenced (Accession no AY388980).

The 212 bp of *pIL 12p35* gene promoter region shows high homology with human promoter region and was found to have the conserved transcription elements. There are some peculiar feautures of the promoter of this gene which is similar to that of humans. Exon 1 of this gene contains the cDNA open reading frame with two potential initiator methionine codons separated by 33 amino acid codons, the region immediately upstream of the first initiator methionine codon contains a TATA like promoter motif (TATAAA) that, if functional, would preclude transcription of this codon. It has been reported that human *IL 12p35* gene produced by lymphoblastoid cells differs from that of the one produced by LPS induced monocyte cells. The former produces mRNA which is 200 bases more than that produced by latter type of cells (Hayes *et al.,* 1998). In the same study the transcription start sites were mapped for

Exon1...GTGAGTAACCGGCCAAGAAAGACTCAGCGCTCGGGCGGGAGGGGTGGCGGCCGGGA
AAGAGGAGGCGGAAATTTGGGACAGACTACTCAGAGACTTGTGCAGAGGAAAAAGAAAGGGGGA
AAGACACTGGGTGCTTACCATGAGCTCCATAGGATGTGGTCTCTTGAATCCTGAAACAGCGCCA
TGAGCTAAATATTATGCCCACTTCACGGATGAGAAACTGAGGCCGCACGGAATGGTTTTCTGCC
CAGGTCCCTGGGCTACAGCTCTAAGAGGACAGTTCTCAGATGGCTTCAAAGCGGGGCTTCGTCA
CTCCACTGTTTCCAAAATACTGGGTTGTTTCACCACCTGGCCTCAGTTTCCCTCCTCTTGAAAT
GGAGACTTCTTCTGGGTGaACCCTTGACTGAACTCACAAGGTGGCCAATGATGGAGAGTTGGCT
TTCAACCCTAGAATAGAATGCCCCCCTTAAAGAACCCAATTCATCCCTTAGCTGTTGAGAAAGA
GTGGAAaaGAGTGGAGGGAGGTTGGTGCCCAGaAGTGGATGGGTGGAATGGCCAGGAGGGATGa
AAGGCAGAGGCAGGCCAGCTCATCCTGCCCCCATTCCCCTCCAG...Exon2

TCTTCAAGTCCTCGTAGATACTGCTAAGGCACAGGGTCTGGATAATATGACAAAATGGGGAAGG
GACCAATGAGATGAGCTAAACACACAACCCCCCCTCACTTACCCTGCCACTGCATTTGAGAMAA
GAGTTTTGTGTCTTAC...Exon4...TAGAAGAAAAAATAATCAGACATCAATGACATGAATT
CATAATATGAGCTGAAACTTTTTTTTTTTTTTTTTTTTCTGACTTAC...Exon5...TAGAG
AAAAAAAAAATCACATCAARGATATTAATTCTTAGTATAAAATTTTCTGATTTTTCTCCTCCAA
CATATCTATTTTCTTATTTTGGAAAAAAAGCACACATAAAATTTACCATCTCAACTAATTTTAA
GTTTATAGTTCAGTAGTGTTAAGTATATTCACACT

Fig. 16. Intron-1, 3, 4 and 5 sequence of *pIL 12p35*.

transcripts produced by different cell lines. These were found to be conserved in porcine sequence also. The start site of lymphoblastoid cells (S2), the cDNA start sites for NKSF and CLMF, the TATA box, the monocyte start site (S1) and the two initiator methionine were found similar to that of humans, these are marked in the fig 17. Moreover, the down stream core promoter element and TF II B Recognition Element (Lagrange *et al.,* 1998) were also found to be present in the promoter region of *pIL 12p35*. In another study by Tone *et al.,* 1996 evidences were provided for the existence of an additional 5' untranslated exon in the mouse *IL 12p35*, however, there is no evidence for the existence of a similar upstream noncoding exon in the porcine gene.

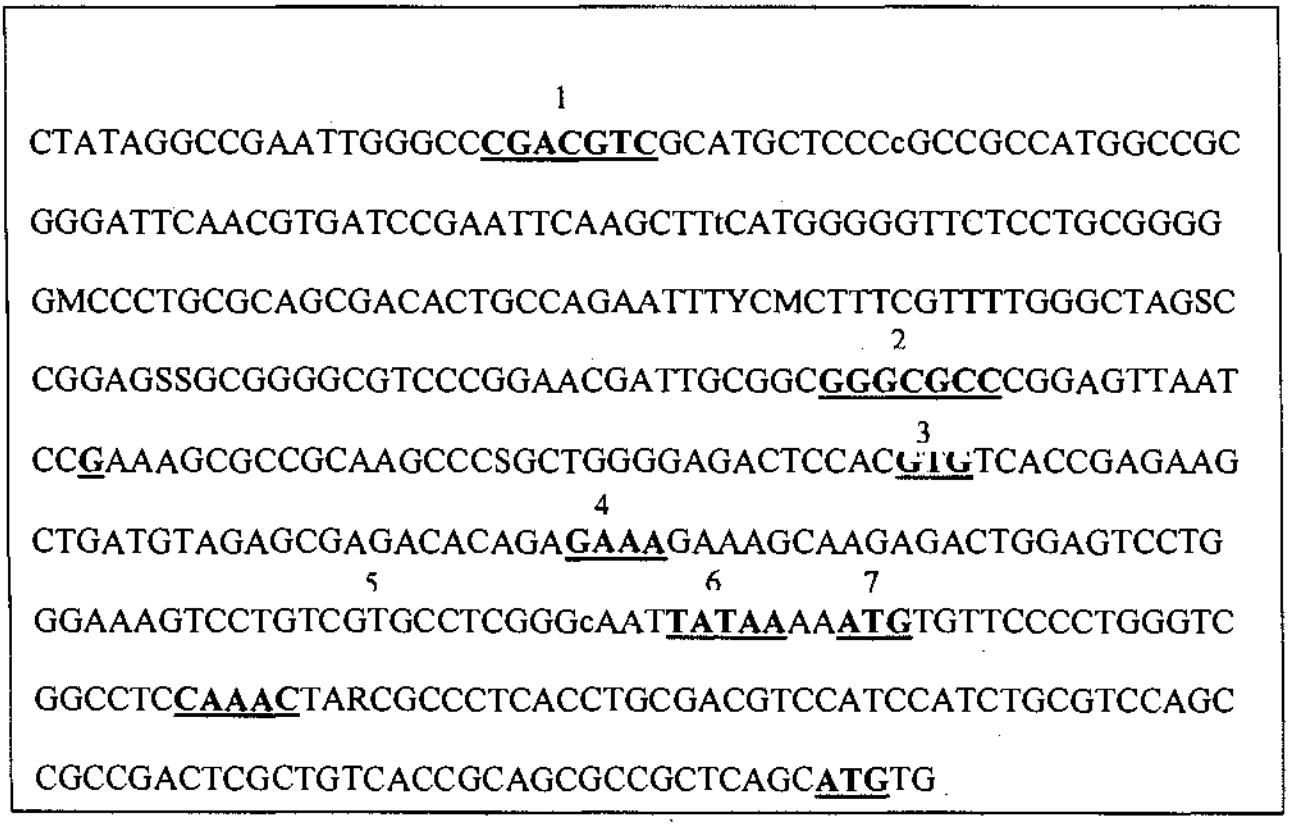


Fig. 17. Promoter region of porcine *IL 12p35* gene.

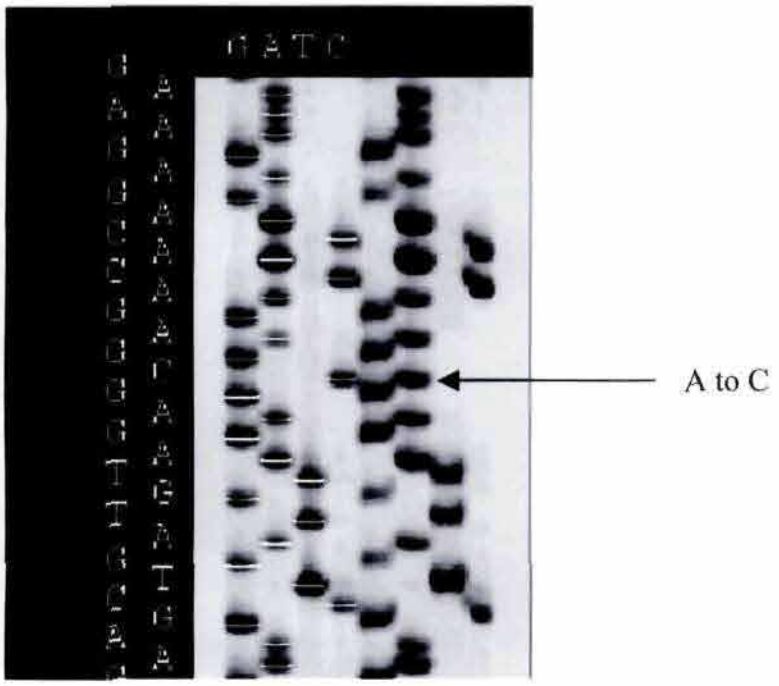
1. DPE: Down stream core promoter element
2. BRE: TF II B Recognition Element (Lagrange *et al.,* 1998)
3. S2 – Lymphoblastoid
4. NKSF cDNA start
5. CLMF cDNA start
6. TATA motif : TATA box or Hogness box, TF II D
7. S1 – Monocyte

4.1.2.1 Polymorphism screening

A 1471 bp cDNA of porcine *IL 12p35* gene has already been sequenced by Foss and Maurtaugh (1997). It contains an open reading frame of 256 amino acids. Interleukin-12*p35* gene has 7 exons and 8 introns similar to that of human *IL 12p35*. In this study, introns 2, 4, 5 and 6 were sequenced. Based on the above sequence information, 3 primer pairs were designed to amplify overlapping PCR fragments of about 400-500 bp from spleen cDNA of one animal from each of the five breeds viz. Duroc, German Landrace, Hampshire, Pietrain, and F1 cross of Duroc x Berlin Miniature Pig by specific the primer pairs. The amplified products were run on 1 % agarose gel and the band was excised to isolate the DNA. All of the PCR fragments were cloned and comparatively sequenced to identify polymorphisms.

No mutations could be detected between the breeds in cDNA. Since no mutations could be detected in exonic region of the gene, introns were screened for presence of single nucleotide polymorphisms (SNPs). The Introns 1, 3, 4 and 5 were 612, 144, 85, and 168 bp, respectively. Intron-4 has a T repeat of 21bp which was also reported to be presented in cattle and sheep (Schmidt *et al.*, 2000), but unlike in cattle and sheep where variation in repeat number has been reported, no polymorphism could be detected between the pig breeds screened in this study. Since no variations could be detected in the introns, promoter region sequence was identified using vectorette PCR and comparatively sequenced among different pig breeds. One SNP could be detected at 72 bases upstream start codon. The SNP detected doesn't have any restriction site and also could not be detected by PCR-SSCP, hence Allelic discrimination assay (Fig. 18) was used to detect the polymorphism. The polymorphism detected was found to be seggregating in the DUMI resource population, but could not be found in any of the commercial breeds screened.

a.



b.

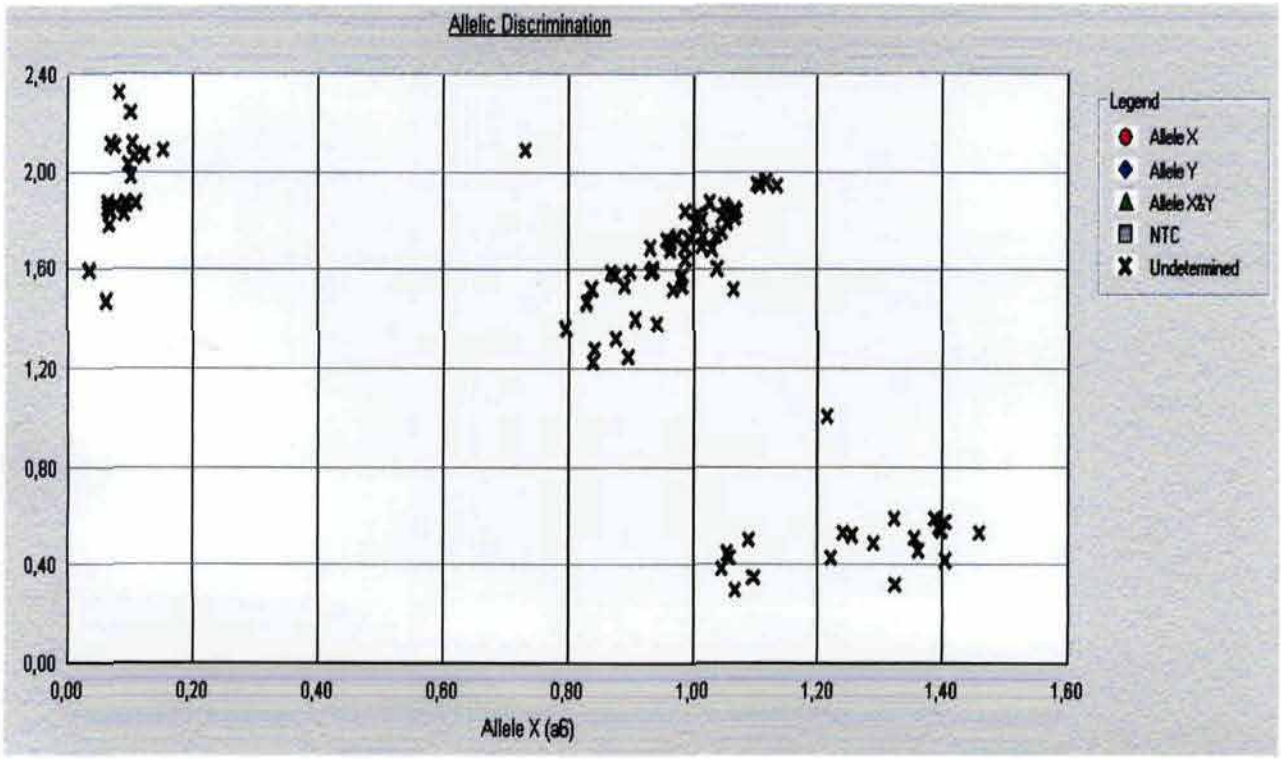


Fig 18. (a) polymorphism A to C of the *pIL 12p35* gene in the promoter region (b) allelic discrimination assay in DUMI resource population.

4.1.2.2 Chromosomal localization of *pIL 12p35*

Mapping using Radiation Hybrid Panel

The *pIL 12p35* was mapped using Radiation Hybrid mapping panel of INRA, France. PCR was carried out using primers *IL12-35-Fw* – gaggcacgacaggactttcca and *IL12-35-Re* - cccggagttaatccgaaagc to amplify genomic DNA for 118 pig-hamster hybrid clones along with a pig and a hamster DNA as a positive control. The PCR products were run on 2 % agarose gel and the data for all the 118 clones (1 or 0 for presence or absence of band) were submitted to IMpRH webserver of INRA, France (http://imprh.toulouse.inra.fr/) to find the relative position of the gene to the markers already mapped. The most significantly mapped marker was found to be SW 1876 (0.92cR and LOD = 3.19) which was assigned to chromosome 13 (Fig. 19) (Alexander *et al.*, 1996).

Linkage Mapping

The data obtained from genotyping Bonn DUMI resource population was used for linkage mapping of the genes. The data was checked for any genotyping errors by using Pedcheck (Version 1.1). Two point linkage analysis was carried out by using CRI-MAP package (Version 2.0). Results confirm the result obtained by RH mapping.

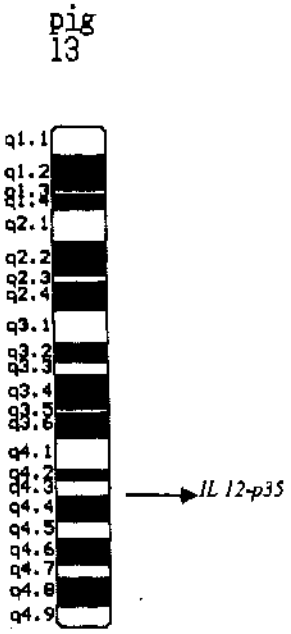


Fig. 19. Localization of *pIL 12p35* gene in Sscr13.

4.1.2.3 Association between *pIL 12p35* genotypes with various immunologcial parameters

The statistical analysis for association between various complement activity traits and genetic markers was performed using the repeated statement in the proc mixed procedure of the SAS program. Four covariance structures of proc mixed repeated statement viz. autoregressive (AR), heterogenous autoregressive (ARH), compound symmetry (CS) and heterogenous compound symmetry (CSH) were fitted for each of the trait and the best fit was used in the model. Statistical model was given in the charpter materials and method. Different factors like dam, sire, litter size, parity, and sex were considered along with genotype and time of vaccination and the factors which was found to be significant was used further in the model. The correlation coefficents between two measures on the same animal at all times were 0.26 and 0.57 for the alternative and classical complement activity traits, respectively. But in case of serum complement C3 protein, correlation was found to be decreasing with increasing length of time interval measures, which ranged from 0.7 between first two measurements to 0.08 between the last two measurements.

The statistical analysis revealed a high significance between *pIL 12p35* markers for classical (CH50) complement activity at different time intervals (Table 18).

Table 18. Effect of *IL 12p35* marker with various complement activity traits (ACH50 and CH50) and serum complement C3 protein).

Source	Immunological traits			
	ACH50	CH50	C3c	HP
IL 12-p35	ns	*	ns	ns
Time	*	*	ns	ns
IL 12-p35 *time	*	*	ns	ns

* - $p<0.05$ ns – Not significant

Association between *pIL 12p35* marker and classical complement activity

The association between *pIL 12p35* markers and classical complement activity traits at different sampling time is given in table 19. The overall mean of CH50 of *TT* genotype animals was 5.39 units/ml less than that of *TG* genotype animals. ($p<0.01$). Moreover, the effect of *IL 12p35* genotypes was found to be significantly different after vaccination of Mycoplasma (time 3), before and after vaccination of Aujeszky disease vaccination (time 4, time 5 and time 6) and also before and after PRRS vaccination (time 7 and time 8) whereas no significant difference between the genotypes could be found at time 1 and time 2 (Fig. 20).

Table 19. Least squares means with standard errors for classical complement activity of each *pIL 12p35* genotype at individual sampling times.

Vaccination	Blood sampling	*IL 12p35* genotype	
		TT	*TG*
Mycoplasma	Time 1	45.06 ± 3.97	45.44 ± 4.94
	Time 2	53.34 ± 3.89	54.07 ± 4.77
	Time 3	55.42 ± 3.85*	50.81 ± 4.62
Aujeszky	Time 4	61.4 ± 3.85	71.84 ± 4.52*
	Time 5	68.16 ± 3.83	81.2 ± 4.5*
	Time 6	63.71 ± 3.85	70.14 ± 4.55*
PRRS	Time 7	64.58 ± 3.87	74.65 ± 4.57*
	Time 8	64.2 ± 3.87	70.8 ± 4.56*
	Overall	59.48 ± 3.49	64.87 ± 3.96*

* - $p<0.05$

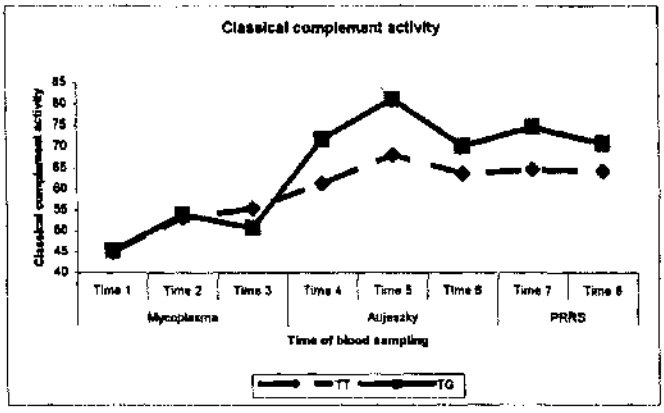


Fig. 20. Grapbical representation of classical complement activity at different time of blood sampling for the 2 *pIL 12p35* genotypes.

Association between *pIL 12p35* marker and alternative complement activity

The association between *pIL 12p35* markers and alternative complement activity traits at different sampling time is given in table 20. The overall mean of AH50 of *TT* genotype animals was 1.81 units/ml less than that of *TG* genotype animals, which was not statistically significant. But, the *pIL 12p35* genotypes was found to be significantly different at after vaccination of Mycoplasma (time 2 and time 3), after vaccination of Aujeszky disease vaccination (time 5 and time 6) and also before and after PRRS vaccination (time 7 and time 8) whereas no significant difference between the genotypes could be found before vaccination of Mycoplasma and Aujeszky (time 1 and time 2) (Fig. 21).

Table 20. Least squares means with standard errors for alternative complement activity of each *pIL 12p35* genotype at individual sampling times.

Vaccination	Blood sampling	*pIL 12p35* genotype	
		TT	*TG*
Mycoplasma	Time 1	53.36 ± 3.29	53.92 ± 3.26
	Time 2	55.34 ± 3.28*	47.62 ± 3.92
	Time 3	57.52 ± 3.26*	50.79 ± 3.95
Aujeszky	Time 4	56.75 ± 3.24	56.28 ± 3.83
	Time 5	58.15 ± 3.26	64.98 ± 3.83*
	Time 6	65.54 ± 3.30	70.11 ± 3.85*
PRRS	Time 7	67.08 ± 3.29	75.86 ± 3.88*
	Time 8	66.41 ± 3.26	75.08 ± 3.82*
	Overall	60.02 ± 2.56	61.83 ± 2.87

* - $p<0.05$

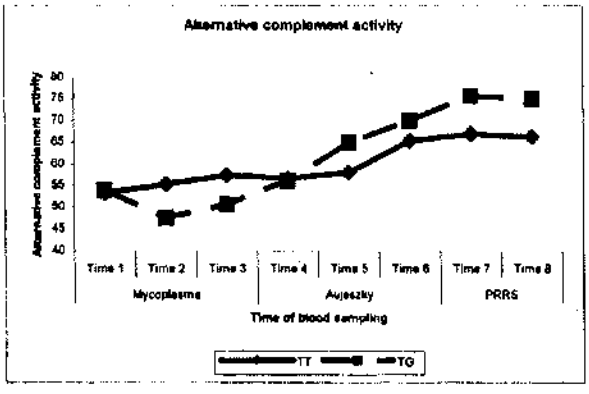


Fig. 21. Graphical representation of alternative complement activity at different time of blood sampling for the 2 *pIL 12p35* genotypes.

Association between *pIL 12p35* marker and C3c protein levels

The association between *pIL 12p35* markers and C3c protein levels at different sampling time is given in table 21. The overall mean of C3c levels of *TT* genotype animals and *TG* genotype animals was 0.19. There was no significiant change of C3c protein levels at different time of blood sampling. The values ranged from 0.17 to 0.21. Though not statistically different, there was an increase in C3c level immediately after vaccination which increased steadily with age (Fig. 22).

Table 21. Least squares means with standard errors for C3c protein values of each *pIL 12p35* genotype at individual sampling times.

Vaccination	Blood sampling	*pIL 12p35* genotype	
		TT	*TG*
Mycoplasma	Time 1	0.17 ± 0.005	0.18 ± 0.006
	Time 2	0.18 ± 0.005	0.18 ± 0.006
	Time 3	0.18 ± 0.006	0.19 ± 0.009
Aujeszky	Time 4	0.19 ± 0.005	0.20 ± 0.006
	Time 5	0.20 ± 0.005	0.21 ± 0.006
	Time 6	0.19 ± 0.006	0.21 ± 0.008
PRRS	Time 7	0.19 ± 0.006	0.19 ± 0.009
	Time 8	0.20 ± 0.006	0.20 ± 0.009
	Overall	0.19 ± 0.005	0.19 ± 0.006

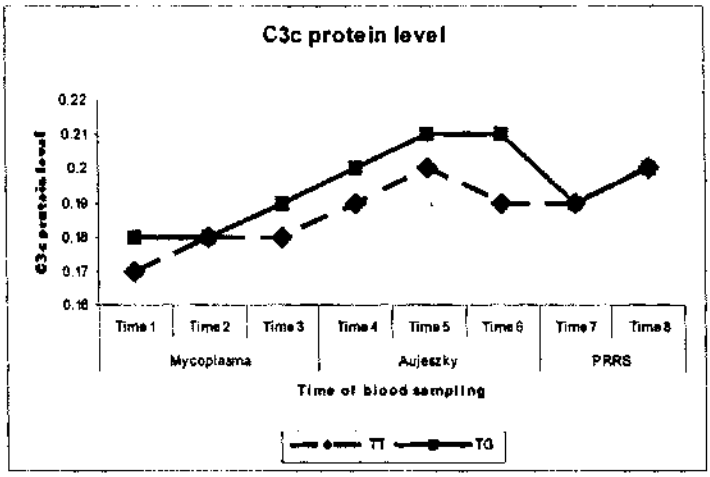


Fig. 22. Graphical representation of C3c protein levels at different time of blood sampling for the 2 *pIL 12p35* genotypes.

Association between *pIL 12p35* marker and Haptoglobin protein levels

The association between *pIL 12p35* markers and Haptoglobin protein levels at different sampling time is given in table 22 and trend is given in fig. 23. The overall mean of Haptoglobin levels of *TT* genotype animals and *TG* genotype animals was 0.50 and 0.47 units/ml respectively. There was no significiant change of Haptoglobin protein levels at different time of blood sampling. There was a significant increase in Haptoglobin level immediately after vaccinations and return back to its normal value after some time. There was also increase in values as age progresses.

Table 22. Least squares means with standard errors for haptoglobin protein levels of each *pIL 12p35* genotype at individual sampling times.

Vaccination	Blood sampling	*pIL 12p35* genotype	
		TT	*TG*
Mycoplasma	Time 1	0.38 ± 0.02	0.36 ± 0.02
	Time 2	0.56 ± 0.03	0.47 ± 0.02
	Time 3	0.37 ± 0.03	0.37 ± 0.02
Aujeszky	Time 4	0.32 ± 0.03	0.30 ± 0.02
	Time 5	0.80 ± 0.03	0.76 ± 0.02
	Time 6	0.58 ± 0.02	0.52 ± 0.02
PRRS	Time 7	0.48 ± 0.02	0.46 ± 0.02
	Time 8	0.51 ± 0.02	0.53 ± 0.02
	Overall	0.50 ± 0.02	0.47 ± 0.02

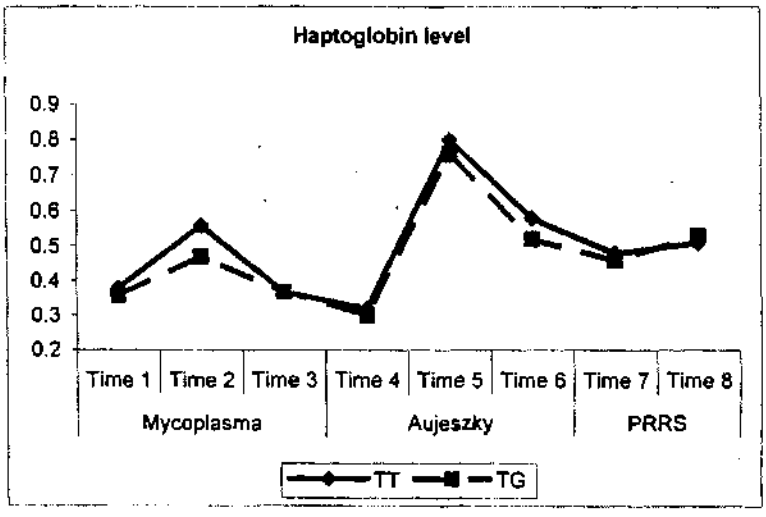


Fig. 23. Graphical representation of haptoglobin levels at different time of blood sampling for the 2 *pIL 12p35* genotypes.

Association of *pIL 12p35* markers with various antibody titres before and after vaccination

Association between *pIL 12p35* markers with various antibody titres (Mycoplasma, Aujeszky, PRRS) were analysed by using 'general linear model' procedure of SAS software (v8.02). Various effects like sire, dam, litter size, parity, sex, etc. were cosidered for the analysis and those which had significant effect on the trait were used for analysis. The results are showed in table 23. The results showed that there is no significant effect of markers on antibody titres before as well as after vaccination for all the vaccination traits.

Table 23. Effect of *pIL 12p35* marker on various antibody titres before and after vaccination.

Source	Immunological traits				
	Mycoplasma pre vac	Mycoplasma post vac	Aujeszky pre vac	Aujeszky post vac	PRRS post vac
pIL 12p35	ns	ns	ns	ns	ns

ns – Not significance

4.2 Complement component *C5*

4.2.1 Sequence identification of porcine complement component *C5* (*pC5*)

Complete porcine *C5* (*pC5*) cDNA was isolated and sequenced in this study. The *pC5* sequence was obtained from liver mRNA by using heterologous primers derived from conserved human, murine and rat sequences and primers derived based on sequence information of ESTs (Accession no. BF713665, D84333 and D84332) of *pC5* gene and the 5' region of the gene was identified by using RACE-PCR technique. Five fragments of sizes ranging from 450 to 1400 bp were obtained. These fragments were cloned in pGEM-T vector and sequenced for complete sequence identification.

In this study 5422 bp of *pC5* were sequenced (Accession no. AY332748). Coding sequence was from 1 to 5031. The sequences from 5032 to 5422 bp were 3' untranslated region. The signal peptide was coded from 1 to 54 bp, beta chain was coded by 55 to 2019 bp and alpha chain is coded from 2032 to 5031. The region 2092 to 2196 coded for anaphylatoxin homologous domain or anaphylatoxin like domain. The region 55 to 1893 coded for N-terminal region of α2 Macroglobulin family protein, and the region 2272 to 4527 coded for protein of C-terminal region of α2-Macroglobulin family. The TATA signal (ATTAAA) was found to be located in 5411 to 5416, similar to that of human *C5* gene. The porcine *C5* gene showed high homology with mammalian *C5* sequence (human, rat and mouse sequences). The complete *pC5* sequence is given below in fig. 24.

```
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TCACTCCTGATTAAAAATTTCCTCTTGAATATGGATGTCAAAGTTTCTTACAAGCATAGAGGTGACTTCTACCAT
TATAAAATTACAGAGAAGAATTTCATTGGGAGGCCAGTAGAGGTGCCTCTCAATGATGACCTTGTTGTGTTCAGT
ACTGGACAGAACAGTGGCTTGGCTACAGTGCATGTAAAAACTGTGGTTCACAAAACCAGTACCTCTGAGGAAGTT
TGCAGCTTTGATTTGAAAATCGAAATCCAGGATATTGAAGCATCCAGCTACAGCAGCTCAGAATACAAGCGCATA
GTGGCCTGTGCCAGCTACAAGCCCAGCAGGGAAGAATCATCATCTGGGTCCTCCCATGCAGTGATGGATATCTCA
CTGCCTACTGGAGTCAATGCAAACGCAGAAGACTTAAGAGCTCTTGTAGAAGGGGTGGATCAACTATTAACTGAT
TATGAAATCAAAGATGGACATGTTATTTGTCAGTTGAACTCGATTCCTTCCAATAATTTCCTCTGTGTTCGATTC
CGGATAGTTGAACTTTTTCAAGTTGGGTTTCTGAGTCCTGCTACTTTCACAGTGTATGAGTACCACAGACCAGAT
AAACAGTGCACCATGTTTTATAGCATGTCCCAGACCAAACTTCAGAAAGTCTGTGAAGGAGTAACGTGCAAATGT
GTTGAAGCTGACCGTGGACAAATGCAGACAGAAGTGGACCTAACAATCTCTGTAAATACTAGGAAAGAAACAGCA
TGTAAACCAGAGATTGCATATGTTTATAAAGTTAAGATCATAGCCATGACTGAAGAAAGTGCTTTTGTTAAGTAC
ACCGCGTCCCTACTGGATGTCTACAAAGCTGGAGAAGCCGTTGCTGAGAAAGGCTCTGAAATCACCTTTATTAAA
AAGACAACCTGTACGAACGCTAACCTCGAAAAAGGAAAACAGTACTTAATTATGGGTAAAGAAGCCCTCCAGATA
AAACATAACTTCAATTTCAAGTACATTTACCCTTTAGATTCCTCAACCTGGATTGAATATTGGCCTACAGACACT
GCTTGTCCATCATGCCAAACATTTTTAGCAAATCTAGATGAGTTTACTGAAGACATCTTTTTAAACAACTGTGAA
AACGCCTAACGAAGGTCAGCTGCATCCAGTTTTCACTATGGCTCTCTGTTGTTGAAGTTTCTTCCTTTTTTTTCA
GCACTCACAGCTGGTCTTATTTGTAAAGCTCACCCTACTTAGAATTAGGGACACTTGCTTTCATTAGAGAATGAT
TTTAAGAGCAGTAACTTTCTGAAATAACATGTCCTTGGGGGACACGGAGACAGACACTCATTCCATGGTTATTGG
ACACCAGAAGCAATAAATTGGCACCCTCCTAAAACCTACCACTCAGGAATGTTTGCCAGGGACTGAAGAATAGCC
CCAACTGAAATGTAGTATCTTNCCAAAACATAGCTTTTGCTTGAAAGAAAATACCAAGGAACAGAATACTGNTCA
TTAAAGCCTGACTTTGCTTTCC

Fig. 24. Complete nucleotide sequence of porcine *C5*.

Predicted protein sequence of *pC5* is 1677 a.a. The first 18 a.a. formed the signal peptide, the amino acids from 19 to 673 forms the β chain and 678 to 1677 forms the α peptide the fragment from 19 to 631 amino acids form N-terminal region of α2 macroglobulin family, fragments from 698 to 732 amino acids forms anaphylatoxin like domain, 758 to 1509 forms C-terminal region of α2 macroglobulin family and the fragment 1549 to 1659 represents the NTR/C345C module (Fig. 25). The predicted protein sequence showed high homology with mammalian protein sequences (78 % with human, 73 % with mouse, 71 % with rat) but has low homology with non mammalian species (38 % with *Cyprinus carpio*). When compared with already existing protein sequences from other mammalian species it forms cluster with human sequence, whereas mouse and rat sequence form another cluster. The result indicates the *pC5* and *hC5* were closely related.

4.2.2 Polymorphism screening

The above sequence information of porcine *C5* was used to identify polymorphism in porcine *C5* gene. 12 overlapping PCR fragments *C5*-01 to *C5*-12 were obtained from liver cDNA from one animal of each of the five breeds viz. Duroc, German Landrace, Hampshire, Pietrain and a F2 animal of DUMI population. All the PCR fragments were cloned and sequenced to identify polymorphisms. Once the polymorphisms were detected, simple PCR based methodologies were established to facilitate large scale genotyping.

Fig. 25. Predicted protein structure of porcine *C5*. The signal peptide, the α and β- chains are represented.

Four single nucleotide polymorphisms (SNPs) could be detected at positions 1044, 1203, 2766 and 3018 (Fig. 27). The SNP at position 1044 was a transversion from A to C, whereas the SNPs at position 1203, 2766 and 3018 were transition mutations from A to G, T to C and A to G, respectively. All the SNPs detected in this study are silent mutations. All the mutations are found in commercial breeds. The polymorphism at position 1044 was found to be segregating in resource population (fig 26).

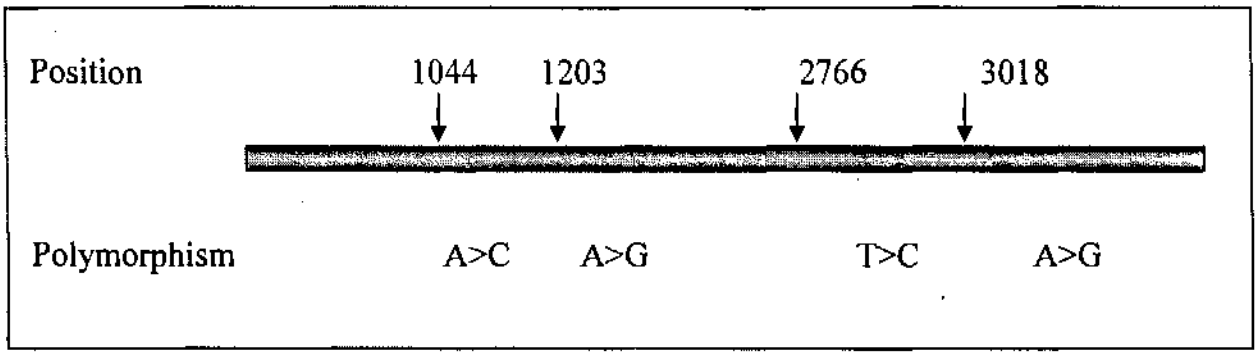

Fig. 26. Summary of polymorphisms in the *pC5* gene.

All the polymorphic sites could be detected by PCR-RFLP (Fig 27). The genotype and gene frequencies of all the alleles were calculated for three commercial breeds viz. German landrace, Pietrain and DE (n= 20 to 25) and Indian native pigs (n= 15) (Fig. 29) and are presented in tables 24 and 25, respectively. The genotype GG at position 3018 was not found in any German commercial animals that were genotyped but was found to be in very high frequency in Indian native pigs.

Table 24. Genotype frequencies of various *pC5* alleles in different breeds.

Breeds	*C5* – 1044			*C5* – 1203			*C5* – 2766			*C5* – 3018		
	AA	AC	CC	AA	AG	GG	TT	TC	CC	AA	AG	GG
German Landrace	0.0	0.07	0.93	0.57	0.26	0.17	0.18	0.61	0.21	0.9	0.1	0.0
Pietrain	0.03	0.27	0.7	0.5	0.4	0.1	0.24	0.57	0.19	0.95	0.05	0.0
DE	0.03	0.27	0.7	0.3	0.13	0.57	0.27	0.64	0.09	1.0	0.0	0.0
Indian Native	0	0	1.0	0	0	1.0	0	0.33	0.67	0.07	0.53	0.40

Table 25. Gene frequencies of various *pC5* alleles in different breeds.

Breeds	*C5* – 1044		*C5* – 1203		*C5* – 2766		*C5* – 3018	
	A	C	A	G	T	C	A	G
German Landrace	0.035	0.965	0.70	0.30	0.485	0.535	0.95	0.05
Pietrain	0.165	0.835	0.7	0.3	0.515	0.485	0.975	0.025
DE	0.165	0.835	0.365	0.635	0.59	0.41	1.0	0.0
Indian Native	0	1.0	0	1.0	0.17	0.83	0.06	0.94

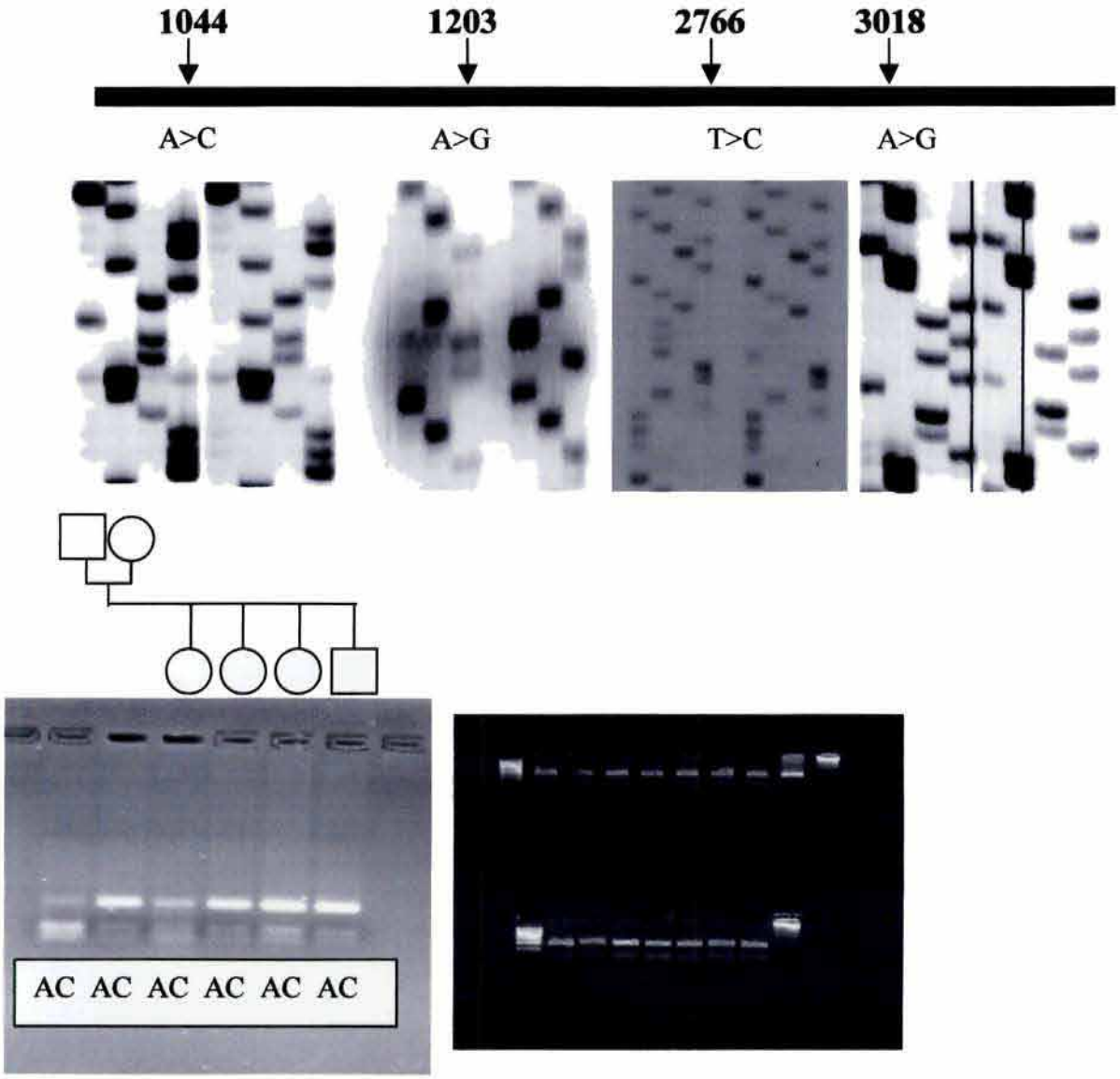

Fig. 27. The polymorphisms of p*C5* cDNA (top). The polymorphism at position 1031 was segregating in the Bonn DUMI resource population (bottom left) and polymorphism were also seen in Indian native pigs (bottom right).

4.2.3 Chromosomal localization of gene

Mapping using Radiation Hybrid Panel

By using radiation hybrid panel of INRA, France, the *pC5* gene was assigned to chromosome 1q1.8 or p2.8-q2.13 (Fig. 28) (Ponsuksili *et al.*, 2001).

Linkage Mapping

The data obtained from genotyping Bonn DUMI resource population was used for linkage mapping of the genes. The data was checked for any genotyping errors by using Pedcheck (Version 1.1). Two point linkage analysis was carried out by using CRI-MAP package (Version 2.0). The results revealed linkage to loci SW1301 with recombination fraction of 0.33 on sex averaged map with an LOD score of 4.51. SW1301 has been assigned to Sscr1q2.13.

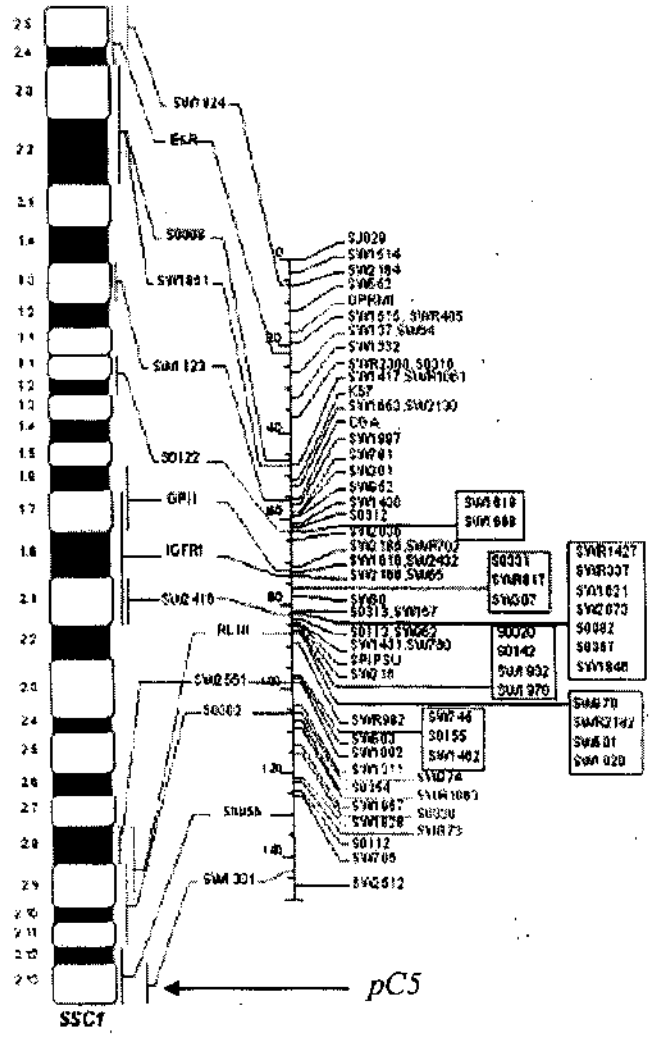

Fig. 28. Localization of *pC5* gene in Sscr1q2.13

4.2.4 Association analysis of p*C5* genotypes with various immunological traits in DUMI resource population

Association between *pC5* markers (AA - *pC5*1/pC5*1,* AC- *pC5*1/pC5*2,* CC - *pC5*2/pC5*2)* at position 1031 which is segregating in the resource population and various immunological traits was carried out in 417 animals of the DUMI resource population. The alternative complement activity, classical complement activity, plasma complement C3 protein concentration, antibody titer against Mycoplasma, Aujcszky and PRRS vaccines, before and after vaccinations were the phenotypes considered for association analysis.

The statistical analysis for association study involving various complement activity traits, C3c protein level and haptoglobin values and *pC5* marker was performed using the repeated statement in the proc mixed procedure of the SAS program (Version 8.1). Four covariance structures of Proc mixed repeated statement viz. autoregressive (AR), heterogenous autoregressive (ARH), compound symmetry (CS) and heterogeneous compound symmetry (CSH) were fitted for each of the trait and the best fit was used in the model. Different factors like dam, sire, litter size, parity and sex were considered along with genotype and time of vaccination and the factors which was found to be significant was used further in the model.

Table 26. Effect of *pC5* marker with various complement activity traits and acute phase proteins.

Source	Immunological traits			
	ACH50	CH50	C3c	HP
pC5	*	*	*	*
Time	*	*	*	*
pC5 *time	*	*	*	*

* - $p<0.05$

Association between *pC5* markers and classical complement activity (CH50)

The association between *pC5* markers and classical complement activity traits at different sampling time is given in table 27 and the trend is given in fig.29. The overall mean of CH50 of *pC5*1/pC5*1, pC5*1/pC5*2* and *pC5*2/pC5*2* animals was 72.41 ± 4.23, 65.06 ± 3.66 and 62.81 ± 7.29 units, respectively. The animals having *pC5*1/pC5*1* were found to have 7.35 units and 9.6 units higher than *pC5*1/pC5*2* and *pC5*2/pC5*2* respectively, which is statistically significant. Moreover there was a significant rise in complement activity levels immediately after mycoplasma and aujeszky vaccinations, but no significant difference existed for prior and after vaccination levels in case of PRRS vaccination.

Table 27. Least squares means with standard errors for classical complement activity of each *pC5* genotype at individual sampling times.

Vaccination	Blood sampling	*PC5* genotype		
		*pC5*1/pC5*1*	*pC5*1/pC5*2*	*pC5*2/pC5*2*
Mycoplasma	Time 1	53.76 ± 5.23[a]	47.76 ± 4.63[b]	49.03 ± 10.26[b]
	Time 2	62.78 ± 5.02[a]	58.80 ± 4.50b	63.57 ± 10.05[a]
	Time 3	59.08 ± 4.87[b]	57.17 ± 4.43[b]	65.03 ± 10.05[a]
Aujeszky	Time 4	77.31 ± 4.73[a]	70.24 ± 4.39[b]	61.56 ± 10.25[c]
	Time 5	92.49 ± 4.74[a]	74.41 ± 4.34[b]	64.91 ± 10.52[c]
	Time 6	78.57 ± 4.81[a]	67.75 ± 4.35[b]	63.59 ± 10.37[b]
PRRS	Time 7	76.30 ± 4.83[a]	72.47 ± 4.35[b]	70.46 ± 10.38[b]
	Time 8	78.99 ± 4.79[a]	71.69 ± 4.35[b]	64.32 ± 10.91[c]
	Overall	72.41 ± 4.23[a]	65.06 ± 3.66[b]	62.81 ± 7.29[b]

means in a row with different superscript are significantly different ($p<0.05$)

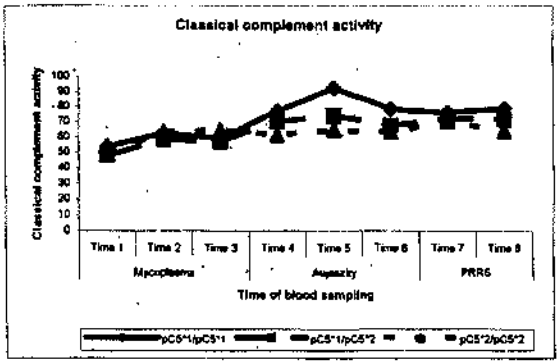

Fig. 29. Classical complement activity levels at different time of blood sampling for the 3 genotypes of *pC5* gene.

Association between *pC5* markers and alternative complement activity (ACH50)

The association between *pC5* marker and alternative complement activity is given below in table 28 and the trend is given in fig.30. The alternative complement activity of *pC5*1/pC5*1* was found to be 5.35 units and 4.25 units lower than the *pC5*1/pC5*2* and *pC5*2/pC5*2* genotype animals, which was found to be statistically significant. The same pattern existed for blood sampling time before and after Mycoplasma vaccination, whereas no significant difference between genotypes existed for blood sampling before and after Aujeszky vaccination, but the alternative complement level tend to decrease for *pC5*2/pC5*2* at time 7 and 8 than the other genotypes.

Table 28. Least squares means with standard errors for alternative complement activity of each *pC5* genotype at individual sampling times.

Vaccination	Blood sampling	*PC5* genotype		
		*pC5*1/pC5*1*	*pC5*1/pC5*2*	*pC5*2/pC5*2*
Mycoplasma	Time 1	55.21 ± 4.68^{b}	59.76 ± 4.40^{b}	71.14 ± 10.27^{a}
	Time 2	53.54 ± 4.64^{b}	64.75 ± 4.26^{a}	66.29 ± 9.85^{a}
	Time 3	55.74 ± 4.57^{a}	63.65 ± 4.28^{b}	79.06 ± 9.85^{c}
Aujeszky	Time 4	61.35 ± 4.50	62.05 ± 4.21	63.72 ± 9.85
	Time 5	65.27 ± 4.48^{b}	71.71 ± 4.25^{a}	61.46 ± 10.63^{b}
	Time 6	76.88 ± 4.53	78.46 ± 4.25	79.35 ± 10.63
PRRS	Time 7	75.39 ± 4.62^{a}	80.27 ± 4.24^{b}	66.92 ± 11.70^{c}
	Time 8	72.89 ± 4.49^{b}	78.40 ± 4.20^{b}	62.30 ± 10.21^{a}
	Overall	64.53 ± 3.63^{a}	69.88 ± 3.13^{b}	68.78 ± 5.47^{b}

means in a row with different superscript are significantly different ($p<0.05$)

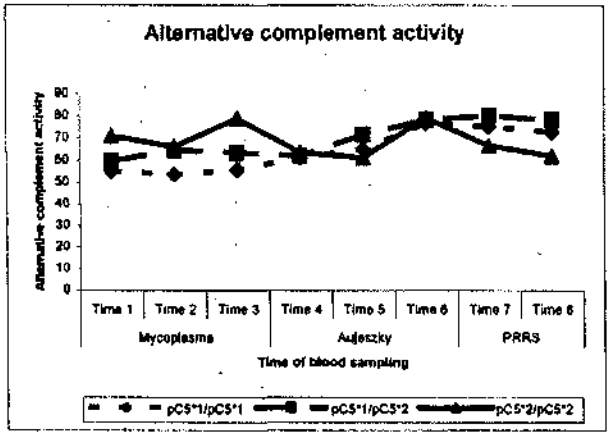

Fig. 30. Alternative complement activity levels at different time of blood sampling for the 3 genotypes of *pC5* gene.

Association between C3c protein level and *pC5* genotypes

Least square means of the C3c protein levels for different genotypes are given in table 29 and the trend is given in fig.31. In case of C3c protein genotype *pC5*2/pC5*2* has 0.014 and 0.009 units higher than *pC5*1/pC5*1* and *pC5*1/pC5*2*, respectively, which is statistically significant. There was a significant increase in C3c level after mycoplasma and aujeszky vaccinations, when compared with prevaccination blood samples, but no significant difference exits between the time of blood sampling before and after PRRS vaccination.

Table 29. Least squares means with standard errors for C3c protein level for each *pC5* genotype at individual sampling times.

Vaccination	Blood sampling	*PC5* genotype		
		*pC5*1/pC5*1*	*pC5*1/pC5*2*	*pC5*2/pC5*2*
Mycoplasma	Time 1	0.178 ± 0.005^{b}	0.173 ± 0.004^{b}	0.187 ± 0.007^{a}
	Time 2	0.187 ± 0.005^{b}	0.181 ± 0.004^{b}	0.192 ± 0.007^{a}
	Time 3	0.189 ± 0.007^{b}	0.196 ± 0.006^{b}	0.181 ± 0.008^{a}
Aujeszky	Time 4	0.194 ± 0.005^{b}	0.202 ± 0.004^{b}	0.223 ± 0.006^{a}
	Time 5	0.203 ± 0005^{b}	0.207 ± 0.004^{b}	0.222 ± 0.006^{a}
	Time 6	0.188 ± 0.00^{b}	0.211 ± 0.006^{a}	0.216 ± 0.006^{a}
PRRS	Time 7	0.195 ± 0.006^{b}	0.196 ± 0.006^{b}	0.202 ± 0.009^{a}
	Time 8	0.198 ± 0.007^{b}	0.199 ± 0.006^{b}	0.214 ± 0.009^{a}
	Overall	0.191 ± 0.004^{b}	0.196 ± 0.003^{b}	0.205 ± 0.004^{a}

means in a row with different superscript are significantly different ($p<0.05$)

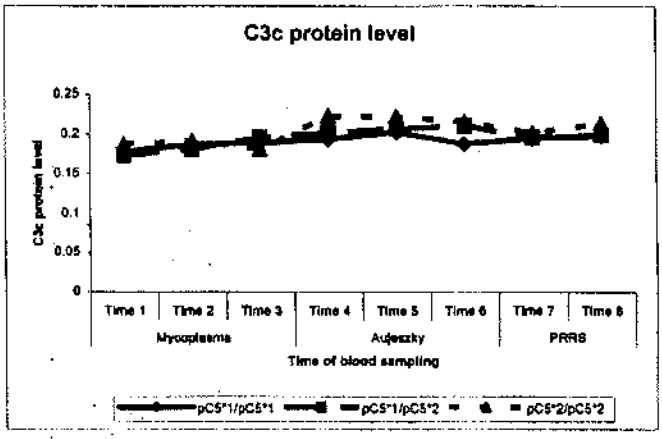

Fig. 31. C3c protein level at different time of blood sampling for the 3 genotypes of *pC5* gene.

Association between haptoglobin protein level and *pC5* genotypes

Least square means of the haptoglobin protein levels for different genotypes is given in table 30 and the trend is given in fig.32. In case of haptoglobin level *pC5*2/pC5*2* genotype was found to be significantly different from the other two genotypes. The *pC5*2/pC5*2* genotype was found to have 0.123 units and 0.088 units higher haptoglobin level than *pC5*1/pC5*1* and *pC5*1/pC5*2* genotypes, respectively. There was a significant increase in haptoglobin level after Mycoplasma and Aujeszky vaccinations than before vaccination samples, wheras no significant change in haptoglobin level was observed before and after PRRS vaccination.

Table 30. Least squares means with standard errors for haptoglobin level for each *pC5* genotype at individual sampling times.

Vaccination	Blood sampling	*PC5* genotype		
		*pC5*1/pC5*1*	*pC5*1/pC5*2*	*pC5*2/pC5*2*
Mycoplasma	Time 1	0.325 ± 0.036^{b}	0.342 ± 0.032^{b}	0.499 ± 0049^{a}
	Time 2	0.519 ± 0.038^{c}	0.552 ± 0.032^{b}	0.669 ± 0.049^{a}
	Time 3	0.386 ± 0.038^{c}	0.451 ± 0.032^{b}	0.591 ± 0.051^{a}
Aujeszky	Time 4	0.323 ± 0.037^{b}	0.378 ± 0.032^{b}	0.438 ± 0.049^{a}
	Time 5	0.726 ± 0.036^{b}	0.715 ± 0.033^{b}	0.778 ± 0.049^{a}
	Time 6	0.610 ± 0.036^{c}	0.678 ± 0.033^{b}	0.719 ± 0.049^{a}
PRRS	Time 7	0.503 ± 0.033^{b}	0.544 ± 0.033^{b}	0.607 ± 0.050^{a}
	Time 8	0.536 ± 0.036^{b}	0.546 ± 0.034^{b}	0.614 ± 0.051^{a}
	Overall	0.491 ± 0.020^{b}	0.526 ± 0.018^{b}	0.614 ± 0.0299^{a}

means in a row with different superscript are significantly different ($p<0.05$)

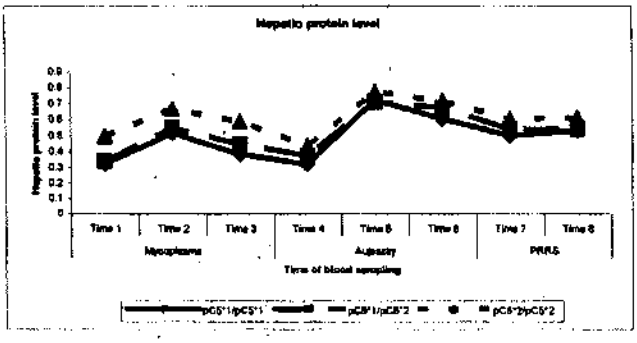

Fig. 32. Haptoglobin levels at different time of blood sampling for the 3 genotypes of *pC5* gene.

Association between *pC5* markers and the antibody titre against Mycoplasma, Aujeszky and PRRS vaccines

Proc mixed of GLM procedure of SAS (version 8.1) was used to analyze the association between *pC5* markers and the antibody titer against Mycoplasma, Aujeszky and PRRS vaccines. The results showed no significant difference between the genotypes both before and after vaccinations (table 31).

Table 31. Effect of *pC5* marker with various antibody titres before and after vaccination.

Source	Immunological traits				
	Mycoplasma pre vac	Mycoplasma post vac	Aujeszky pre vac	Aujeszky post vac	PRRS post vac
pC5	ns	ns	ns	ns	ns

ns – No significance

5 Discussion

General or innate disease resistance in farm animals is important in animal breeding as it could provide increased protection against many ubiquitous facultative pathogens which are involved in causing considerable economic losses. Economic losses from disease of farm animals have been estimated to account for 10 – 20 % of the total production costs (Müller and Brem, 1991). Viewing its economic potential many researches have been carried out in the recent past, for improvement of disease resistance in animals.

Innate response of immune system involves complement, acute-phase proteins and cytokines. The early events of complement activation can be triggered by any of the three pathways viz. classical, alternative or lectin pathway. Irrespective of the source of activation, the outcome is the generation of a number of immunologically active substances. For eg, a proteolytic clevage fragment of complement component C3, the C3b molecules, becomes deposited on the surface of microorganisms. This event enhances phagocytosis of the microbe, because phagocytic cells have cell surface receptors for C3b. The complements C3a, C4a and C5a cause the release of inflammatory mediators from mast cells. C5a also acts as a powerful neutorphil chemoattractant.

Complement component C5 plays a major role in complement system as being the first of the many components of the terminal pathway and also the C5a which is a cleaved product of C5 by C5 convertase is an anaphylatoxin which is an essential component in mediating inflammatory process. C5 mediates many potent inflammatory and cytolytic events after proteolytic activation by complement convertase enzymes (Haviland *et al.,* 1991). Homozygous C5 deficient individuals were found to have propensity for severe recurrent infections, particularly to *Neisserial* species (Ross and Densen, 1984). Sera from C5 deficient individuals lack bactericidal activity and have greatly impaired ability to induce chemotaxis (Rosenfeld *et al.,* 1976). Complement factor 5 was identified as a susceptible locus for allergic asthma (Karp *et al.,* 2000).

Thus C5 plays a crucial role in natural resistance against invading microbes/ pathogens.

Cytokines constitute another group of soluble mediators that are involved in innate disease resistance. They are biological messengers, known under various synonyms based on their actions and the source from which it comes from, like lymphokines, interleukins, interferons, etc. which plays a pivotal role in immune system as it forms a cellular information exchange network via cell-cell interactions. Among the biological messengers, interleukin 12 (*IL 12*), plays an essential role in the interactions between the innate and adaptive arms of immunity. *IL 12* deficiency was associated with recurrent episodes of pneumococcal pneumonia with sepsis and other infections in the absence of fevers (Haraguchi *et al.*, 1998) and *Salmonella* enteritis disseminated infection in children (Altare *et al.*, 1998). *IL 12* β promoter heterozyogsity contributes to asthma severity in children (Morahan *et al.*, 2002). A new disease susceptibility locus, *IDDM1*8, located near the interleukin-12 (*IL 12*) *p40* gene, was also found (Morahan *et al.*, 2001). In Japanese families, mite-sensitive asthma was found to have a significant linkage to chromosomal region 5q31-q33 near the interleukin 12-β locus (Yokouche *et al.*, 2000). The above studies represent *IL 12* as an important gene in disease resistance.

Considering the importance of the complement component *C5* and Interleukin 12 in innate immune resistance, this study was carried out to identify the complete cDNA sequence of *C5* as it was not sequenced, to detect the polymorphism in *C5* and *IL 12* genes and to carry out association analysis between polymorphic markers detected and various immunological parameters measured in resource population which was based on a cross of Duroc and Berlin Miniature pig (DUMI resource poplulation).

5.1. Interleukin 12 (*IL 12*)

Interleukin 12 (*IL 12*) plays an essential role in the interactions between the innate and adaptive arms of immunity. Previously called as natural killer cell stimulatory factor (NKSF) (Kobayashi *et al.*, 1989) and cytotoxic lymphocyte maturation factor (CLMF)

(Stern *et al.,* 1990), it is primarily produced by phagocytic cells, B cells, dendritic cells and possibly other accessory cells when encountered with infectious agents. *IL 12* acts on T-cells and NK cells by enhancing generation and activity of cytotoxic lymphocytes and inducing proliferation and production of cytokines, especially IFN-γ. Moreover, it favors the differentiation of precursor Th cells into Th1 effectors (Trinchieri, 1995).

IL 12 deficiency was associated with recurrent episodes of pneumococcal pneumonia with sepsis and other infections in the absence of fevers (Haraguchi *et al.,* 1998) *IL 12* deficiency in a child was associated with *Salmonella* enteritis disseminated infection (Altare *et al.,* 1998). *IL 12* β promoter heterozyogsity contributes to asthma severity in children (Morahan *et al.,* 2002). A single base change in the 3′ UTR showed strong linkage disequilibrium with the T1D susceptibility locus. The *IL 12* β 3′ UTR alleles showed different levels of expression in cell lines (Hall *et al.,* 2000). A new disease susceptibility locus, *IDDM18*, located near the interleukin-12 (*IL 12*) *p40* gene, was also found (Morahan *et al.,* 2001). In a genome wide scan for QTL in Japanese families for mite-sensitive asthma a significant linkage to chromosomal region 5q31-q33 near the interleukin 12-β locus was found (Yogouche *et al.,* 2000). These studies demonstrated that *IL 12* as an important gene in disease resistance.

Interleukin 12 is a heterodimeric cytokine which plays an important role in innate and adaptive immune response. Biologically active *IL 12* is a 70 kD heterodimer consisting of 40 kDa β protein (*p40*) and 35 kDa α protein (*p35*), linked by disulphide bonds. These two proteins were structurally unrelated and were produced by two different genes which were located in different chromosomes.

5.1.1 Interleukin *12p40* (*IL 12p40*)

A 2124 bp cDNA of *pIL 12p40* gene has already been sequenced by Foss and Maurtaugh, 1997 (Accession No. U08317) who found that porcine *IL 12p40* subunit was 85 % and 70 % homologous to human and murine species, respectively. Cysteine

residues present in the porcine *IL 12* subunits were conserved in both human and murine sequences, consistent with the finding that disulfide bonds were required for bioactivity. Potential N-linked glycosylation sites and cysteine residues were conserved between all three species. The 3' untranslated region contains 12 AUUUA repeats (Foss and Murtaugh, 1997) which is responsible for rapid degradation of mRNA. *pIL 12p40* contains 7 exons similar to that of humans. In this study intron-4 of the gene was amplified, cloned and sequenced. It is about 561 bp long which was comparable with that of human sequence (542 bp) in length.

5.1.1.1 Identification of polymorphism in *pIL 12p40*

For uncovering polymorphism in gene of interest, animals could be chosen, by 2 ways. First by selecting the animals having extreme phenotypic traits. This approach has been used successfully for health status and some production traits in farm animals such as porcine stress syndrome (Fujii *et al.,* 1991), bovine leukocyte adhesion deficiency (Shuster *et al.,* 1992), double muscling in cattle (Grobet *et al.,* 1997; McPherron and Lee 1997). Another approach is to select divergent breeds to detect the polymorphisms. Many polymorphism in candidate genes have been found with this approach, like porcine estrogen receptor (Rotschild *et al.*,1996), heart fatty acid binding protein (Gerbens *et al.,* 1997), complement component C3 (Wimmers *et al.,* 2003), etc.

In this study, the second strategy was used successfully for screen for polymorphism in *pIL 12p40, pIL 12p35* and *pC5* genes by comparing individual breeds of pig (Hampshire, Duroc, Landrace, Large white and a F2 cross-bred animal of DUMI population).

No polymorphism could be detected in exonic region of the *pIL 12p40* gene, the dearth in polymorphism in exons could be due to high level of conservation of gene and also indicated its importance in immune response. Supporting evidence was found in a study involving human *IL 12p40* gene, where eleven polymorphic regions were found, but only in intronic regions (Huang *et al.,* 2002). However some difference could be

found between the cDNA sequence obtained in this study and the sequence already submitted by Foss and Maurtaugh, 1997. Anyhow all the changes observed were silent mutations. Moreover, breed of the pig which was used to sequence by them was not reported, hence could not be confirmed. Since no polymorphism could be detected in exons, intron 4 was sequenced to find the polymorphism. Two polymorphic sites could be detected, both were transition mutations. Various reports of genetic polymorphism in this gene were found, mostly in humans (Huang *et al.*, 2002; Morahan *et al.*, 2002; Noguchi *et al.*, 2001; Altare *et al.*, 1998;) and were reported to be associated with asthama susceptibility (Morahan *et al.*, 2002), Salmanella enteritis disseminated infection (Altare *et al.*, 2001), etc. indicating the importance of *IL 12p40* in immune regulation. Hence the genetic markers detected in this study were used to study association with various immunological parameters measured in DUMI resource population.

Since sequencing the whole resource population for genotyping was laborious, time consuming and expensive and also due to difficulty in finding the heterozygous, simple, efficient, faster and cost-effective methodologies like PCR-RFLP and PCR-SSCP were established to detect the polymorphisms for genotyping the resource population and commercial breeds of pigs. PCR-RFLP was used to detect the polymorphism in INT4-437C>T and PCR-SSCP was used to detect the polymorphism in INT4-196A>G. The two polymorphic sites of *IL 12 p40* gene were found to be linked in the DUMI resource population and were found to be segregating in 3 genotypic pattern AC/AC, AC/GT and GT/GT. The polymorphic site at position 437 could be seen in Indian native pigs also. Though genetically distant from the German commercial breeds, the presence of polymorphic site in Indian breed was a surprising finding suggestive of more detailed study in this aspect.

5.1.1.2 Immunological traits

Because of the importance of the complement system in host resistance through direct lysis of target cells and support of other mechanisms of the innate and adaptive immune system, haemolytic complement activity, both alternative and classical

pathway, were studied, which reflect the *in vitro* capacity of the complement system of a given sample. In addition, the C3c serum concentration was measured, which indicates *in vivo* complement activation during the acute-phase response. In parallel, the serum concentration of Haptoglobin (HP) the most important acute-phase protein in pigs was studied in a subset of animals. Since antibody titre value measures the adaptive immune response of an animal, the values were measured before and after each of the three vaccination viz. Mycoplasma, Aujeszky and PRRS to carry out association with genetic markers. The values of the haemolytic complement activities, C3c protein level and HP acute phase protein levels were already presented.

The haemolytic complement activity of pigs (between 6 and 20 weeks old) under the vaccination program of this experiment were altered from 49.50 units/ml prior vaccination to 63.06 units/ml after three vaccinations in alternative pathways and from 39.17 to 59.63 units/ml in classical pathways. In previous studies it was reported that the porcine haemolytic complement activity changed from 8 to 33 units/ml from alternative complement activity (Ish *et al.*, 1993; Renshaw and Gilmore 1980) and from 3.6 to 210 units/ml for classical pathway (Barta and Hubbert 1978, Komatsu *et al.*, 1998). These values showed high individual variation in haemolytic complement activity. It has been reported that the variations of complement activities were associated with many factors such as age (Arya and Goel 1992), infection or vaccination (Mallard *et al.*, 1989a; Wimmers *et al.*, 2003) and nutritional status (Sakamoto *et al.*, 1981) as well as genetics (Komatsu *et al.*, 1998; Wimmers *et al.*, 2003).

In this study, it was found that there was short-term elevation of acute phase protein levels (C3c and HP) immediately after vaccination, which declines to normal state in later stage of blood sampling. Vaccination leads to an acute phase response measurable by an increased serum level of HP (Asai *et al.*, 1999; Rekitt *et al.*, 2001; Wimmers *et al.*, 2003) and C3c level (Wimmers *et al.*, 2003) in pigs. There was also a slight elevation of hemolytic complement activity immediately after Mycoplasma and Aujeszky Disease vaccination, probably due to interaction of Mh lipopolysaccharide with C1 (Bredt *et al.*, 1977) and also because Aujeszky immunization activates both

pathways, as described for other α-herpesviridae (Mayes *et al.,* 1984). Mean values of complement activity is comparable to previous studies (Mallard *et al.,* 1989).

5.1.1.3 Association between porcine *IL 12p40* markers and immunological traits

Considering the importance of *pIL 12p40* in innate immune system, genetic polymorphism of *pIL 12p40* was analysed for association with haemolytic complement activity, C3c serum levels, HP levels and antibody titre values against Mycoplasma and Aujeszky disease vaccination. Since the measurements are made in the same animals at 8 different times, repeated statement function of SAS program was used. Various covariance structures viz, autoregressive (AR), heterogeneous autoregressive (ARH), compound symmetry (CS) and heterogeneous compound symmetry (CSH) were investigated to fit a specified covariance structure with the data of each trait. Since the data contains unequal class number for each of the genotype the data was corrected by using Tukey-Krammer correction factor. Due to small number of animals in genotype *IL 12p40*2/IL 12p40*2* the animals in this group were not considered for analysis

In the present study *pIL 12p40* markers were found to be in association with complement activity traits. The genotype *IL 12p40*1/IL 12p40*1* was found to have 7.26 units/ml higher classical complement activity (CH50) than *IL 12p40*1/IL 12p40*2* and 4.86 units/ml lower alternative complement activity (ACH50). Significant effects of *pIL 12p40* genotypes with haemolytic complement activity traits indicated that the genetic variation of *pIL 12p40* was associated on haemolytic complement activity. Since the polymorphism was in intron region of the gene, it could not be the causative polymorphism. The effect might be due to its linkage with other polymorphism that might be in regulatory region of *pIL 12p40* gene which has not been covered in this study. Though there were reports about polymorphism in *pIL 12p40* gene in humans which was found to be in association with various diseases, no reports were available about its influence on complement activity traits, hence could not be compared or contrasted.

There was no significant difference between the genotypes for the C3c level and HP level, indicating that *pIL 12p40* has no influence on the acute phase protein levels. It may be due to the fact there may be other polymorphism in the promoter region which was not covered in this study, since the level of *IL 12p40* influence the level of *IL 12*. So further study should be carried out to uncover polymorphic region in promoter region and its influence on expression level.

Similarly, there was no significant difference between the genotypes for the antibody titre values both before and after vaccination, indicating that *pIL 12p40* did not have any influence on antibody titre values. This is in contrast to the reports that *IL 12* could be used as an adjuvant to increase the immune response (Portielje *et al.*, 2003). Since the number of animals taken in this study for this analysis were less in number, further study with more number of animals was suggested to draw a definite conclusion about the influence of *IL 12* in antibody titre values.

5.1.1.4 Chromosomal localization of *pIL 12p40*

The *pIL 12p40* was mapped using Radiation Hybrid mapping panel of INRA, France. The most significantly mapped marker was found to be SW 262 (40.28 cR and LOD = 10.59) which was assigned to chromosome 16 (Rohrer *et al.*, 1996). Moreover, two point linkage mapping result analysis by using CRI-MAP package from genotyping data from Bonn DUMI resource population revealed the position of this gene was in Sscr 16q11-q12. The two finding supported each other, further these results also agreed with the current comparative maps, with the pig gene having been assigned to q arm of chromosome 16 and the corresponding human orthologue to 5q31-q33.

5.1.2 Interleukin *12 p35 (IL 12p35)*

A 1471 bp cDNA of *pIL 12p35* gene has been sequenced by Foss and Maurtaugh, 1997. It contains an open reading frame of 256 amino acids. The predicted amino acid sequence of the porcine *IL 12p35* was compared with that of human and murine. Both the human and porcine sequences had an additional upstream start codon in frame

which would generate a protein with an additional 34 amino acids at the amino terminal end.

These potential additional amino acids are not required for biological activity of recombinant human protein and the region was only 21% homologous between swine and humans. Nonfunctional upstream start codons were identified in about 10% of mRNA messages. In addition, the downstream translation start site conformed to a Kozak sequence (CANCAUGGC) for optimal initiation wheras the upstream site does not. The predicted porcine amino acid sequence had an additional three amino acids and 86% homology to human *IL 12p35* and 7 additional amino acids and 66% homology to murine *IL 12p35*. The first 25 amino acids of the porcine sequence formed a typical signal peptide. Potential N-linked glycosylation sites and cysteine residues were conserved between al three species. The 3' untranslated region contains eight AUUUA repeats that were associated with rapid mRNA degradation in a number of genes. (Foss and Murtaugh, 1997)

Interleukin-12*p35* gene has 7 exons and 8 introns similar to that of human *IL 12p35*. In this study introns 1, 4, 5 and 6 were completely sequenced. Intron-5 has a single sequence repeat (T repeat of 21 bp) which was also reported to be presented in cattle and sheep (Schmidt *et al.*, 2000), but unlike in cattle and sheep where variation in repeat number has been reported, no polymorphism could be detected between the pig breeds screened in this study. Moreover no QTL for disease resistance in this region was also reported.

The promoter region of the *IL 12-p35* has been identified by using Vectorette PCR technique. A 212 bp of 5' promoter region was identified, cloned and sequenced. The 212 bp of *pIL 12p35* gene promoter region showed high homology with human promoter region and was found to have the conserved transcription elements. There were some peculiar features of the promoter of this gene which is similar to that of humans. Exon 1 of this gene contains the cDNA open reading frame with two potential initiator methionine codons separated by 33 amino acid codons, the region immediately upstream of the first initiator methionine codon contains a TATA like

promoter motif (TATAAA) that, if functional, would preclude transcription of this codon. It has been reported that human *IL 12p35* gene produced by lymphoblastoid cells differs from that of the one produced by LPS induced monocyte cells. The former produced mRNA which was 200 bases more than that of produced by latter type of cells (Hayes *et al.*, 1998). In the same study the transcription start sites were mapped for transcripts produced by different cell lines. These were found to be conserved in porcine sequence also. The start site of lymphoblastoid cells (S2), the cDNA start sites for NKSF and CLMF, the TATA box, the monocyte start site (S1) and the two initiator methionine were found similar to that of humans. Moreover, the down stream core promoter element and TF II B Recognition Element (Lagrange *et al.*, 1998) were also found to be present in the promoter region of *pIL 12p35*. In another study by Tone *et al.*, 1996 evidences were provided for the existence of an additional 5' untranslated exon in the mouse *IL 12p35*, however, there was no evidence for the existence of a similar upstream non-coding exon in the porcine gene.

5.1.2.1 Identification of polymorphism in *pIL 12p35*

The comparative sequence analysis of the gene for identification of SNPs in the coding region of the gene, doesn't reveal any polymorphism. It showed the importance of the function of this gene and also its evolutionary importance. Since no polymorphism could be detected in exons, introns were sequenced, which surprisingly didn't have any polymorphic changes. Intron –IV of *pIL 12p35* gene was found to contain a poly T stretch (21 bp) which was similar to that of cattle and sheep (Schmidt *et al.*, 2000), but unlike the sheep and cattle gene, there were no changes in the number of repeats. Since introns were found to have no polymorphic sites, promoter was sequenced and was found to have a T-G transversion at 72 bp upstream start codon.

Since the polymorphic site doesn't have any restriction site, allelic discrimination assay using florescent probes were used to identify the genotypes. The SNP assay contains two primers for amplifying the sequence of interest and two TaqMan® MGB probes for distinguishing between two alleles. Each TaqMan MGB probe contained a reporter dye at the 5′ end of each probe. VIC ™ dye was linked to the 5′ end of the

Allele 'G' probe, 6-FAM ™ dye was linked to the 5′ end of the Allele T probe and a minor groove binder (MGB) in both the probe. This modification increases the melting temperature (Tm) without increasing probe length (Afonina *et al.*, 1997; Kutyavin *et al.*, 1997), which allowed the design of shorter probes. This resulted in greater differences in Tm values between matched and mismatched probes, which produced more accurate allelic discrimination.

During PCR, each TaqMan ® MGB probe annealed specifically to a complementary sequence between the forward and reverse primer sites. When the probe was intact, the proximity of the reporter dye to the quencher dye resulted in suppression of the reporter fluorescence primarily by Forster-type energy transfer (Förster, 1948; Lakowicz, 1983). AmpliTaq Gold® DNA polymerase cleaved only probes that are hybridized to the target. Cleavage separated the reporter dye from the quencher dye, which resulted in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence was complementary to the probe and was amplified during PCR. Thus, the fluorescence signal generated by PCR amplification indicated which alleles were present in the sample. Mismatches between a probe and target reduce the efficiency of probe hybridization. Furthermore, AmpliTaq Gold DNA polymerase is more likely to displace the mismatched probe rather than cleave it to release reporter dye. The detail of SNP assay is given below in fig 33.

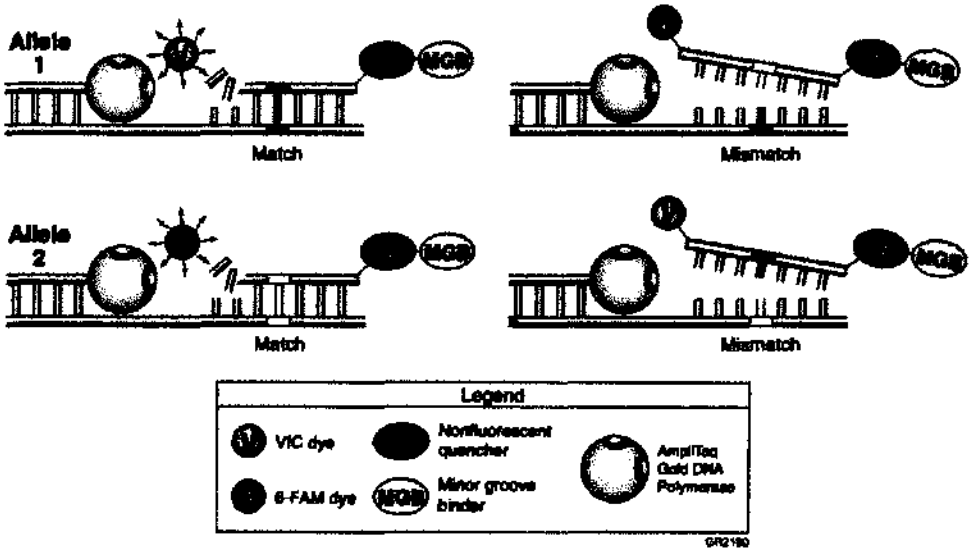

Fig. 33. Overview of SNP assay using TaqMan probe.

5.1.2.2 Association of *IL 12p35* polymorphism with immunological traits

Considering the importance of *IL 12p35* in complement system, genetic polymorphism of *IL 12p35* was analysed for association with haemolytic complement activity, C3c serum levels, HP levels and antibody titre values against mycoplasma and aujeszky disease vaccination. Since the measurements were made in the same animals at 8 different times, repeated statement function of SAS program was used. Various covariance structures viz, autoregressive (AR), heterogeneous autoregressive (ARH), compound symmetry (CS) and heterogeneous compound symmetry (CSH) were investigated to fit a specified covariance structure with the data of each trait. Since the data contained unequal class number for each of the genotype the data was corrected by using Tukey-Krammer correction factor. Since the number of GG homozygote animals was very less, they were excluded from the analysis.

In this study, the TT genotype animals were found to have significantly lower level of classical complement activity level than TG genotype animals, indicating that the polymorphism was associated with classical complement activity. It was found recently that the complement activation products and receptors were biologically relevant regulators of IL 12 production. Complement activation fragments appeared to be able to both augment (*C5*a.*C5*aR) and suppress (*C5*:*C5*aR; C3b:CD46; C3bi: CR3) monocyte/macrophage production of IL 12. (Karp and Karp, 2001). Present findings also supports the above report. But there was no significant difference between the genotypes for alternative complement activity traits.

Moreover, there was no significant difference between the genotypes for acute phase proteins C3c protein and Haptoglobin levels. The result indicates there was no association between the *pIL 12p35* genotype and acute phase protein levels.

5.1.1.3 Chromosomal localization of *pIL 12p35*

The *pIL 12p35* gene has been mapped to Sscr13 by linkage mapping using RH-mapping panel of INRA, France. The most significantly mapped marker was found to

be SW 1876 (0.92cR and LOD = 3.19) which was assigned to chromosome 13 (Alexander *et al.*, 1996). The result was confirmed by two point linkage mapping using CRI-MAP package using data obtained by genotyping of DUMI resource population. The two finding supported each other, further these results also agreed with the current comparative maps, with the pig gene having been assigned to q arm of chromosome 13 and the corresponding human orthologue to 3q12-q13.2.

5.2 Porcine complement component *C5* (*pC5*)

The complement system is a highly regulated and complex set of interacting proteins involved in removal of invading microbes. It circulates in an inactive form and become sequentially activated in response to activation to a microbe. It is one of the important effector systems of innate immunity, is present in lower as well as in higher animals (Sunyer *et al.*, 1998). The complement system interacts critically with other mechanisms of innate immunity as well as with adaptive immunity to enhance host defence (Song *et al.*, 2000).

Complement component *C5* mediates many potent inflammatory and cytolytic events after proteolytic activation by complement convertase enzymes (Haviland *et al.*, 1991) and plays a major role in complement system as being the first of the many components of the terminal pathway. C5a which is a cleaved product of C5 by C5 convertase is an anaphylatoxin which is an essential component in mediating inflammatory process. C5 deficient individuals display a propensity for severe recurrent infections, particularly to *Neisserial* species (Ross and Densen 1984). Sera from C5 deficient individuals lack bactericidal activity and have greatly impaired ability to induce chemotaxis (Rosenfeld *et al.*, 1976). Complement factor 5 was identified as a susceptible locus for allergic asthma (Karp *et al.*, 2000). Thus *C5* plays a crucial role in natural resistance against invading microbes/ pathogens.

Mammalian C5 is synthesized as a single chain preproform, which is cleaved into its mature form composed of two disulfide-linked chains during secretion (Nilsson *et al.*, 1975). C5 is a member of the thioester-containing protein family including C3, C4 and

α2-macroglobulin (α2M). In the human genome, *C3, C4* and *C5* are paralogous genes (Kasahara 1998). It has been postulated that *C5* diverged first, on the basis of a limited number of partial sequences available for the *C3/C4/C5* family (Hughes 1994). To date, the complete primary structure of *C5* has been determined in humans (Lundwall *et al.*, 1985), mice (Wetsel *et al.*, 1987) and in lower vertebrates rainbow trout (Nonaka *et al.*, 1981), the gilthead sea bream (Franchini *et al.*, 2001) and common carp (Kato *et al.*, 2003).

Since complement component *C5* has the potential to be the candidate mechanism for improved general immune response, this study was carried out to identify the complete cDNA sequence of porcine *C5*, to detect SNPs in the gene and to carry out association analysis between *C5* markers and various immunological parameters measured in F2 animals of a pig resource population based on cross of Duroc and Berlin miniature pigs (DUMI resource population).

5.2.1 Sequence identification and analysis of porcine *C5* gene

The complete porcine cDNA was obtained in this study which spanned about 5422 bp. The sequence was obtained by designing primers based on the information of 2 ESTs of pig similar to that of human *C5* sequences and also based on the conserved region of *C5* sequences in other mammalian species. The 5' region was identified by using RACE-PCR technique.

Sequence analysis revealed that the signal peptide was coded from 1 to 54 bp, beta chain was coded by 55 to 2019 bp and alpha chain was coded from 2032 to 5031. The region 2092 to 2196 coded for anaphylatoxin homologous domain or anaphylatoxin like domain. The region 55 to 1893 coded for N-terminal region of α2 Macroglobulin family protein and the region 2272 to 4527 coded for C-terminal region of α-2 Macroglobulin family. The more ubiquitous polyadenylation signal ATTAAA was found in *pC5*, unlike murine *C5* which has rare polyadenylation signal CATAAA (Westel *et al.*, 1987). The porcine and mammalian *C5* cDNA showed high similarity

(75 to 87 %), whereas with non mammalian species it showed very low identitiy (36 to 40 %). The sequence comparision of *pC5*, *pC3* (accession no. AF154933) and paritial sequence of *pC4* (Trakaljool, personal communication) supports the postulate made by Hughes 1994 that *C5* diverged first as C3 and C4 forms a single cluster.

The predicted protein is one amino acid longer than that of human and mouse *C5* and is βα orientation similar to the other mammalian species. The molecular weight of *pC5* is 188 kDa similar to that of human *C5*. Moreover, when compared with *C5* protein sequences of other mammals, it forms cluster with human whereas, mouse and rat *C5* form another cluster indicating that mouse and rat *C5* diverged earlier than human and porcine *C5* (Fig. 34).

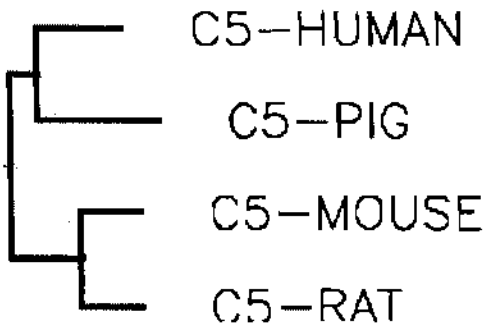

Fig. 34. Predicted protein sequence of *C5* when compared with other mammalian *C5* sequences revealed that human and porcine forms one cluster while mouse and rat forms other.

The functional domains were highly conserved, though the anaphylatoxin domain inspite of its functional similarity showed only 70 % similarity with its human counterpart. The conservation of functional domains indicated the functional similarity of the proteins which explains the cross reactivity of *C5*a between species. The predicted protein sequence in this study is in agreement with the earlier report of porcine anaphylatoxin like domain sequence (Lundwall *et al.*, 1985). The anaphylatoxin domain of porcine *C5* was analysed using the Motif scan prosite (http://hits.isb-sib.ch/cgi-bin/PFSCAN). The results are shown in Fig 35. The results

confirmed the high conservation of the domain. When *C5* anaphylatoxin region of porcine protein was compared to other species, mouse and rat sequences showed an additional 3 amino acids in the amino terminus end, which was absent in porcine sequence. It has been postulated that these three amino acids may greatly enhance the functional activity of the molecule by stabilizing the helical conformation of the critical C terminal region of the molecule (Greer, 1985).

Fig. 35. Anaphylotoxin domain profile as seen in Motif scan prosite.

Several investigators have previously indicated that the β-cysteinyl-α-glutamyl thiol ester present in C3, C4 and α2 macroglobin was not present in *C5* (Law *et al.,* 1980; Janatova and Tack, 1981). The present sequence analysis revealed that *C5* lacks the cysteine residue required for the thiol ester formation but otherwise showed extensive homology (47 %) with porcine C3 sequence. In case of mouse the absence of a thiol ester bond in *C5* is the result of single amino acid differences rather than the deletion or substitution of a larger structural element (Wetsel *et al.,* 1987), which was similar in case of pig (Fig 36).

Porcine C3:- P S G C G E Q N M I G M T P

Porcine *C5*:- P K G N A E A E L M S I V P

Fig. 36. Thiol ester domains of porcine C3 and *C5* aligned. Amino acids which are identical are underlined.

5.2.2 Identification of polymorphism in *pC5*

Uncovering polymorphism in *pC5* was done by comparing individuals of different pig breeds. Sequence comparision of *pC5* between 5 breeds of pigs revealed four polymorphic sites at positions 1044A>C, 1203A>G, 2766T>C and 3018A>G in *pC5* cDNA. The mutation at first position was a transversion mutation, whereas the others were transition mutations. All the SNPs are silent mutations. The sequencing result showed no variation from already published EST sequences. The anaphylatoxin like domain of pig which was already reported also found to be similar to the present sequence. Though there were reports of functional polymorphism in humans (Karp *et al.*, 2000), mouse (Wetsel *et al.*, 1990), there were no reports about the polymorphism study in pigs. Moreover, the polymorphism study that were reported in humans invariably leads to *C5* deficiency and an increased susceptibility to diseases (Wetsel *et al.*, 1990, van den Berg *et al.*, 1991; Karp *et al.*, 2000) indicating the importance of *C5* in immune function. In this study all the four polymorphic sites were detected in the commercial breeds of pigs, whereas only two sites (2766 and 3018) were found to be present in Indian native pigs. Moreover, the polymorphic position at 3018, the genotype GG was not found in commercial breeds, whereas it was found to be very high in Indian native pigs. This may be due to the fact that they were genetically distant. Indian native breeds of pigs are known for its disease resistance ability, since it grows in a hardy environment with minimal management. Hence further studies could be directed towards obtaining phenotypic traits in this \pigs and association study could be carried out. One of the SNP (1031A>C) was found to be segregating in the resource population.

Since sequencing the whole resource population and commercial population for genotyping is laborious, time consuming and high cost and also due to difficulty in finding the heterozygous, simple, efficient, faster and cost-effective methodology like PCR-RFLP was established to detect the polymorphisms for genotyping the resource population and commercial breeds of pigs. All the polymorphic sites could be detected by using PCR-RFLP technique.

5.2.3 Association of *pC5* polymorphism with immunological traits

Considering the importance of *C5* in complement system, genetic polymorphism of *pC5* was analysed for association with haemolytic complement activity, C3c serum levels, HP levels and antibody titre values against Mycoplasma and Aujeszky vaccinations. Since the measurements are made in the same animals at 8 different times, repeated statement function of SAS program was used. Various covariance structures viz, autoregressive (AR), heterogeneous autoregressive (ARH), compound symmetry (CS) and heterogeneous compound symmetry (CSH) were investigated to fit a specified covariance structure with the data of each trait. Since the data contained unequal class number for each of the genotype the data was corrected using Tukey-Krammer correction factor.

In this study the genotype AA was found to have higher classical complement activity and lower alternative complement activity than the other two genotypes. The significant effect of *C5* genotype with both alternative and classical complement activities indicated that genetic variation of *C5* is associated with haemolytic complement activity. Since the polymorphic site is a silent mutation it could not be the causative polymorphism. The effect might be due to its linkage with other polymorphism which may be in regulatory region of *C5* that were not covered in this study. Total serum haemolytic complement activity was undetectable in *C5* deficient individuals (Peter *et al.,* 1981), indicating that *C5* infact influences the haemolytic complement activity. Hence it could be assumed that variation in haemolytic complement activity in this study might be due to the difference in the expression level of the 3 genotypes.

Similarly in case of acute phase protein and C3c protein level the genotype CC was found to have significantly higher level of HP and C3c levels. The significant effect of *C5* genotypes with acute phase protein levels indicated that genetic polymorphism of *C5* is infact associated with acute phase protein levels. As in the above case the polymorphic site may not be causative one as it is a silent mutation and the effect may

be due to its linkage with the causative polymorphism which may be in regulatory region which is not covered in this study.

There was no association of *C5* genotypes with antibody titre against Mycoplasma and Aujeszky vaccinations. The result indicated the *C5* has no influence on antibody titre values, which might be due to that the antibody titre values are indicator of more of an adaptive immune response than natural immunity. *pC3* polymorphism was also found to influence the haemolytic activity in pigs (Wimmers *et al.*, 2003) indicating that haemolytic complement activity depends not only on *C5*, but on many other genetic factors as well.

5.2.4 Chromosomal localization of the gene

The linkage mapping analysis of *pC5* was in agreement with the earlier mapping result by RH and somatic hybrid panel mapping (Ponsuksili *et al.*, 2001). Moreover the results also agrees with the current comparative maps, with the pig gene having been assigned to q arm of chromosome 1 and the corresponding human orthologue is in 9q34 (Wetsel *et al.*, 1988).

5.3 Future prospects for investigation of candidate genes for general immune responsiveness in pigs

Selection for improved general disease resistance in farm animals is highly desirable not only for animal welfare, but also reducing the losses due to disease and hence is an excellent way of improving production efficiency. The cytokines and complement system are considered as important for general host defence mechanism. The present study revealed that the polymorphisms in porcine *C5* gene affect the phenotypic variation of complement activities in the F2-DUMI resource population. Since the polymorphic sites were silent mutations, association of the mutation with complement activity traits and acute phase proteins indicated that the mutation was in linkage with the actual causative mutation. Hence further study should be carried out towards

identification of polymorphic site in promoter region and also towards expression pattern of different polymorphic animals.

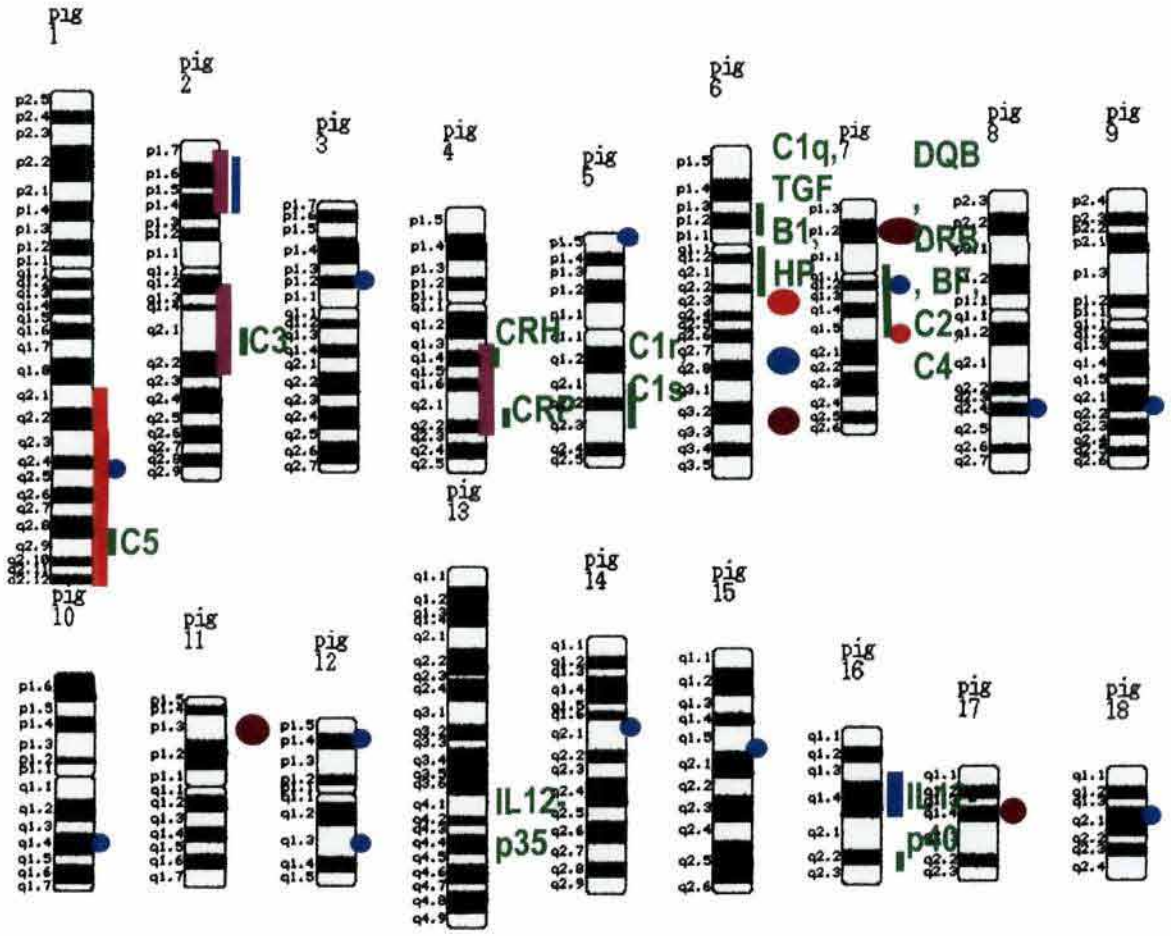

Fig. 39. An ideogram showing the assignment of the porcine component complement, MHC, cytokines their regulation factors and complement receptors genes. Purple colour indicates complement activity, blue colour indicate C3c level and HP level and red colour indicate antibody titre value

In fact, the complement activities are associated with many genes, located on several chromosomes. Therefore the next study of candidate genes for complement activity in pigs should be focused to the other complement components, complement receptors and also complement regulator genes as listed in Figure 39. Out of these genes, only some were analysed for the nucleotide sequence and assigned to pig chromosomes (Peelman et al. 1991; Peelman et al. 1996a; Peelman et al. 1996b; Garrido et al. 1998; Maher et al. 1998; Nakajima et al. 1998; Thomsen et al. 1998; Pérez de la Lastra et al.

1999: Agah et al. 2000; Agah et al. 2001). Although there are little information on most complement genes in pigs, they were localized based on human-pig comparative map (http://www.toulouse.inra.fr/lgc/pig/-compare/compare.htm) as shown in Figure 39. In addition to the candidate genes study for complement activity, the genome wide scanning should be applied as an alternative strategy to indicate the strong QTL regions that affect the complement activity for selecting other candidate genes in this DUMI resource population.

Not many attempts have been made so far to analyse candidate genes for the general immune response in infected pigs. For molecular analysis, several methodologies (e.g. linkage mapping or QTL-mapping, EST-mapping, comparative map, candidate gene) have provided a way to find out the nucleotide polymorphisms that cause phenotypic variation (disease resistance or susceptible loci).

Recently, the pioneering studies have shown QTLs for porcine immune capacity (Edfors-Lilja et al. 1998, Edfors-Lilja et al. 2000) as well as the QTLs and ESTs for pseudorabies and PRRS virus (Reiner et al. 2002, Wang et al. 2001, Zhang et al. 1999). More recently, the nano-technology was shown to have a potential for the genetic dissection of complex traits in the immune response genes of infected animals. Of this technology, oligonucleotide micro arrays or gene chips were used to monitor the genome-wide expression profile of the general immune response genes and a large numbers of unknown functional genes in bacterial or fungi infected *Drosophila* (De-Gregorio et al. 2001, Irving et al. 2001).

All these approaches, as mentioned above, provide the opportunity to identify potential candidate genes for the general immune response traits. In these ways, one can make a significant contribution in animal breeding to select the best genes or alleles that correspond to the general immune responsiveness or specific disease in pigs. These methodologies can be used to improve health status of animals, reduce the need of chemicals and antibiotics in farm animals to produce higher quality products as well as to improve the animal welfare. Ultimately, animal breeding goals for the sustainable livestock production will be achieved.

Owing to the importance of Interleukin-12 and Complement component C5 in immune function, the present study was carried out to investigate porcine *IL 12* and porcine *C5* genes as a candidate for complement activity and immune responsiveness in pigs.

Interleukin-12 is a heterodimeric cytokine consisting of glycosylated subunits of p35 and p40 proteins linked by disulphide bonds. These two subunits are produced by 2 different genes located in 2 different chromosomes. The sequences of these 2 genes in pigs were already identified (Accession no's: U08317 and L35765). Based on the existing sequence information, primers were designed and the two genes were screened for polymorphism by comparing the sequence between 5 different breeds of pigs Duroc, German landrace, Hampshire, Pietrain and an F2 animal of a resource population based on a cross of Duroc and Miniature pig (DUMI resource population).

The F2 animals of DUMI resource population (n = 457) were immunized with Mycoplasma - Mk, Aujeszky - ADV and PRRS vaccines at 6, 14 and 16 weeks of age, respectively. Blood samples were taken immediately prior to immunization (day 0) and on day 4 and 10 after Mk and ADV vaccination and day 10 after PRRS vaccination. For measuring classical complement activity (CH50), sensitized sheep red blood cells (sRBC) were used to measure the haemolysis and for alternative complement activity (AH50) rabbit red blood cells were used. The C3c and HP concentration in serum were measured by immunonephelometrically using a Behring-nephelometer system. The hemolytic complement activity in the alternative pathway ranged between 41.5 ± 28.3 units/ml and 72.2 ± 37.4 units/ml, at different time interval samples, there was a significant increase of AH50 value after Aujeszky vaccination. Classical complement activity values ranged between 48.4 ± 23.1 and 95.1 ± 35.7 at different time intervals of blood collection and showed a significant increase after Mycoplasma and Aujeszky vaccination. C3c concentration ranged from 0.162±. 0.08 to 0.198 ± 0.09 and HP concentration from 0.33 ± 0.21 to 0.79 ±.23, both the acute phase proteins showed significant increase in values immediately after vaccination.

In case of *pIL 12p40* gene, no polymorphism could be detected between the animals in the exonic region. The dearth in polymorphism in coding region indicated

the high conserved nature of the gene and its biological importance. Hence intron 4 of this gene was sequenced (Acc no AY388985). It is about 561 bp which was comparable with human sequence. Two polymorphic site could be detected in intron 4 namely INT4-437C>T which could be detected by PCR-RFLP and INT4-193A>G which could be detected by PCR-SSCP. Both the polymorphism could be seen in commercial breeds of pigs and was also found to be segregating in the resource population. Both the polymorphisms were linked in the resource population. These two SNPs were found to be segregating in 3 haplotypic patterns (*AC/AC-(p40-1)*, *AC/GT (p40-2)* and *GT/GT (p40-3)*). The number of homozygotes for *p40-3* genotype was very small and was hence not considered for the statistical analysis. The statistical analysis for complement activity traits and acute phase proteins were performed using the repeated statement in the proc mixed procedure of the SAS program. Different factors like dam, sire, litter size, parity, and sex were considered along with genotype and time of vaccination. Interaction effect between genotypes and also between genotypes and time of vaccination were also studied. In case of p*IL-12p40*, the genotypes were different with respect to both complement activity traits and haptoglobin level, whereas no significant difference could be found for C3c levels. There was no genotype interaction between the two genes for both complement activity traits and C3c protein levels, whereas significant interaction effect could be found for haptoglobin levels. Association between *pIL 12p40* markers with various antibody titres (Mycoplasma, Aujeszky and PRRS) were analyzed by using 'general linear model' procedure of SAS software (v8.02). The results showed that there is no significant effect of markers on antibody titres before as well as after vaccination for all the vaccination traits. Moreover the gene has been mapped to SSC 16 using Radiation hybrid mapping panel and was confirmed by linkage mapping analysis using the data from DUMI resource population.

In case of IL12p35 gene no polymorphism could be detected in exon region of the gene. The dearth in polymorphism in exon indicates the high conserved nature of the gene. Hence to detect polymorphism in the introns, introns 1, 3, 4 and 5 were sequenced (Accession no's AY388981, AY388982, AY388983 and AY388984). Surprisingly no polymorphism could be detected in these introns. Intron-4 has a poly T stretch of 21 bp similar to that of cattle and sheep. But unlike cattle and sheep no

polymorphism in repeat number could be detected in pigs. Hence promoter region of the gene was detected using Vectorette method. 212 bp of promoter region (Accession No AY388980) was identified which shows many putative features similar to that of humans. A transversion mutation (A>C) could be detected at 72 bp upstream start codon. TaqMan assay using real time PCR was carried out to genotype commercial population and DUMI resource population. The polymorphism was found to be segregating in the resource population.

The p*IL-12p35* polymorphic site was found to be segregating in three genotypic patterns in the resource population (TT (p35-1), TG (p35-2) and GG (p35-3)). The number of homozygotes for *p35-3* genotype was very small for different complement traits and hence not considered for the statistical analysis. Association analysis was performed using repeated statement of proc mixed of SAS software. The statistical analysis revealed a high level of significance between *pIL 12p35* markers for classical (CH50) complement activity at different time intervals. Though no significance could be found between the 2 genotypes for alternative complement activity (AH50), there was a significant effect of time of vaccination as well as time and genotype interaction. No significance between the genotypes as well as between different time intervals could be found for serum C3c level and haptoglobin values. Associations between pIL 12p35 markers with various antibody titers (Mycoplasma, Aujeszky and PRRS) were analyzed by using 'GLM' procedure of SAS software (v8.02). The results showed that there is no significant effect of markers on antibody titers before as well as after vaccination for all the vaccination traits. The gene has been mapped to SSC13 using radiation hybrid panel of INRA, France and was confirmed by linkage mapping using the data from DUMI resource population.

In case of complement component C5, since no sequence informations are available, complete *pC5* cDNA sequence spanning 5422 nucleotides including 390 untranslated residues which precedes the poly A tail (Accession no. AY332748) was sequenced. The polyadenylation signal ATTAAA was found between 5399 and 5405 bp, similar to that of human C5 gene. The porcine C5 gene shows high homology with mammalian C5 sequence (human, rat and mouse sequences). Predicted protein sequence of *pC5* is 1677 amino acids in length, which is one amino acid longer than human C5 and is in βα orientation similar to other mammalian species. Analysis of

porcine protein C5 sequence revealed that the first 18 amino acids form the signal peptide, fragment from 19 to 673 forms the β chain and 678 to 1677 forms the α peptide. Functional domain within the β region is a α2 macroglobulin family N-terminal region from 19 to 631 amino acids and the functional domains within the α regions are anaphylatoxin like domain from 698 to 732 amino acids, α2 macroglobulin family C-terminal region from 758 to 1509 and the NTR/C345C module from 1549 to 1659 amino acids. The predicted protein sequence showed high homology with mammalian protein sequences (78 % with human, 73 % with mouse, 71 % with rat) but has low homology with non mammalian species (38 % with *Cyprinus carpio*). When compared with already existing protein sequences from other mammalian species it forms cluster with human sequence, whereas mouse and rat sequence form another cluster.

The *pC5* gene was screened for polymorphism by comparative sequencing of 5 different breeds of pigs. Four polymorphic sites were found in *pC5* cDNA. One at position 1043 a transversion from A to C and the others are transition mutations A>G, T>C and A>G at positions 1222, 2760 and 3032, respectively. All the SNPs are silent mutations. The polymorphism 1043A>C was found to be segregating in the DUMI resource population as 3 genotypic pattern (*AA, AC, CC*), whereas the other SNPs 1222A>G, 2760T>C and 3032A>G were found only in commercial breeds of pigs. The genotype frequencies for all the polymorphic positions for the commercial breeds and Indian native breeds were calculated, the SNPs 1222A>G, 1043A>C and 2760T>C were found to be in Hardy-Weinberg equilibrium in commercial breeds, whereas 3032A>G was not found to be in Hardy-Weinberg equilibrium.

Analysis of variance was carried out using procedure "Mixed" and 'repeated' statement of the SAS software package (SAS System for Windows, Release 8.02) to investigate effects of different *pC5* genotypes on AH50, CH50, C3c protein and HP concentration. The animals having *AA* genotype were found to have 7.35 units and 9.6 units higher ($P<0.05$) classical complement activity (CH50) than *AC* and *CC* genotypes, respectively. The alternative complement activity of *AA* animals was found to be 5.35 units and 4.25 units lower ($p<0.05$) than the *AC* and *CC* genotype animals. In case of C3c protein, genotype *CC* has 0.014 and 0.009 units higher

concentrations than *AA* and *AC*, genotypes, respectively, which is statistically significant. The *CC* genotype was also found to have 0.123 units and 0.088 units higher ($P<0.05$) HP level than *AA* and *AC* genotypes, respectively. Analysis of variance also showed significant effects of age, sire, dam and parity in addition to the genotypes both for hemolytic complement activities and acute phase protein levels.

GLM procedure of SAS was used to analyze the association between *pC5* markers and the antibody titer against Mk and ADV. The ADV prevaccination values of three genotypes *AA, AC* and *CC* are 0.436 ± 0.047, 0.416 ± 0.043 and 0.439 ± 0.050 units/ml, respectively which after vaccination increases to 0.664 ± 0.062, 0.608 ± 0.056 and 0.644 ± 0.066 units/ml, respectively. In case of Mk before vaccination it was 0.461 ± 0.059, 0.392 ± 0.046 and 0.308 ± 0.083 units/ml which increased to 0.679 ± 0.064, 0.637 ± 0.050 and 0.739 ± 0.090 units/ml for the 3 genotypes. There was a significant increase in overall antibody titer values after vaccination, but there was no significant difference for the antibody titer between the genotypes.

Two point linkage analysis using CRI-MAP revealed linkage of *pC5* to loci SW1301 with recombination fraction of 0.33 on sex averaged map with an LOD score of 4.51. SW1301 has been assigned to Sscr1q2.13.

Mini Abstract

Considering the importance of Interleukin -12 and complement component C5 (*C5*) in innate immunity, the present study was carried out to identify polymorphism in these genes and to genotype a resource population based on cross of Duroc and Berlin Miniature pig (DUMI resource population) of pig, German commercial breeds of pigs and Indian native pigs.

Interleukin 12 (*IL 12*) is a heterodimeric cytokine consisting of 2 glycosylated subunit of p35 and p40 produced by two different genes located in 2 different chromosomes. The genes were screened for polymorphism and association analysis was carried out with various immunological parameters (Classical and alternative complement activities, Haptoglobin level) measured in F2 animals of DUMI population. Two SNPs were detected in intron 4 of *IL12p40* at positions 193 (A to G) and 437 (C to T) and one SNP in promoter region (T to G) of *IL12p35* was identified. All the three SNPs were found to be segregating in the DUMI resource population. Association analysis between SNPs and the immunological parameters was performed using repeated measures mixed model analysis. Significant effect of *IL-12* genotypes with complement activity traits and Haptoglobin levels were found but no significant difference between them could be found for the C3c level. The *IL12p35* and *IL12p40* genes were mapped to Sscr 13 and Sscr16q11-q12, respectively by using IMpRH mapping panel and was confirmed by linkage mapping analysis in DUMI resource population. Gene and genotype frequencies were calculated for the SNPs of *IL12p40* genes in 3 German commercial breeds and Indian native pigs, whereas the polymorphism in promoter region of *IL 12p35* was not detected in commercial breeds of pigs.

In case of porcine *C5* (*pC5*), the complete cDNA of *pC5* was sequenced, screened for single nucleotide polymorphisms (SNPs) and association analysis with various immunological parameters was carried out. 5422 bp of *pC5* cDNA was sequenced which codes for 1677 amino acid precursor C5. Four polymorphic sites were detected one of which was segregating in the DUMI population in 3 genotypic pattern *AA*, *AC* and *CC*. Association analysis between SNPs and the immunological parameters was carried out using repeated measures mixed model analysis. The homozygote *AA* was found to be significantly different from other two genotypes with respect to complement activity traits, whereas, the genotype *CC* was found to be significantly different from other genotypes for acute phase protein levels. The *pC5* gene was mapped to Sscr 1q2.13 by linkage mapping which confirms the earlier reports of RH mapping. All the four polymorphic sites were found in 3 German commercial breeds of pigs, whereas only 2 polymorphic sites were found in Indian native pigs indicating that these are genetically different.

Association of *IL12* and *C5* genes with various immunological parameters suggests that these genes are candidate genes for disease resistance in pigs.

लघु सारांश

इन्टरल्यूकिन-12 और कोम्पालिमेन्ट साधक सी -5 की महत्वता स्वभाविक मुक्ति में समझते हुये प्रस्तुत अनुसन्धान में इनके जीनों की बहुरुपता का विश्लेषण सुअर के कास डयूरोक और वरलिन मिनिचर इूमी आवादी के सुअर), जर्मन सुअरों की व्यवसायिक जातियों और भारतीय पैदाइशी सुअरों पर अध्ययन किया गया।

इन्टरल्यूकिन -12 एक साइटोकाइनिन है जो दो भिन्न ग्लिकोसहलेर की सहायक ईकाई पी-35 और पी-40 से मिलकर बनती है। यह दो विभिन्न जीन जो कि दो भिन्न गुणसूत्रों पर पाये जाते है, इन ग्लिकोसइर्लर को बनाते है। इन जीनों का विश्लेषण बहुरुपता और मिलान विश्लेषण जिसमें विभिन्न इम्नोलोजिकल मापक द्वारा एफ-2 डूमी आवादी में किया गया। आई एल-12 पी-40 मे दो एक न्यूम्योराइड बहुरुपता इसके चौथे इन्टान के 193 और 467 स्थान पर पायी गयी तथा एक बहुरुपता आई एल 12 पी-35 के प्रवर्तक भाग में पायी गयी। एक न्युम्योटाइर बहुरुपता और इमनोलोजिकल मापक का संयुक्त विश्लेषण पुनरुक्ति परिमाण संयुक्त प्रतिरुप द्वारा किया गया। आई एल-12 के जीनोराइप के साथ कोम्पलीमेन्ट तीवता गुण और हप्टोग्लोवलिन समता में अर्थपूर्णता पायी गयी पर सी-3 सी समता में कोई अर्थपूर्णता नहीं थी। जीन और जीनोटाइप आवृत्तियों की गणना के लिए एक न्युम्लपीटाइर बहुरुपता द्वारा आइ.एल-12 पी-40 जीनो में तीन जर्मन व्यवसयिक जातियों और भारतीय पैदाइशी सुअरो मे की जबकि आइ.एल-12 पी-35 के प्रवर्तक भाग में कोई बहुरुपता नही पायी गयी।

सुअरों के पी.सी-5 के सी.डी.एन.ए.का पूर्ण सीम्युन्स किया गया तथा एक न्युम्लेयाटाइड बहुरुपता और विभिन्न इमनोलोजिकल मापांक के साथ संयुक्त अध्ययन किया गया। इसका सी.डी.एन.ए.5422 वेस पेयर का था जिससे 1677 अमीनो एसिड की प्रोटीन बनती है। डूमी आवादी में चार बहुरुपता पायी गयी जो कि जीनोटाइप ए.ए.,ए.सी और सी.सी बनाती है। इसमे समयुग्मजी ए.ए दूसरे जीनाटाइप से कोम्पलीमेन्ट तीव्रता के सम्बन्ध में अर्थपूर्ण भिन्न था जबकि तीव रूप प्रोटीन समता में दूसरे जीनोटाइप, जीनोटाइप सी सी से अर्थपूर्ण भिन्न थे। सभी चार बहुरुपक स्थान तीन जर्मन व्यवसायिक जातियों में पाये गये जबकि केवल दो बहुरुपक स्थान भारतीय पैदाइशी सुअरों में पाये गये यजो यह बतांते है कि यह अनुवंशिक भिन्न है। प्रस्तुत अध्ययन यह बताता है कि सुअरो के रोग प्रतिरोधक में आइ.एल-12 और सी-5 जीन एक याचक जीन है।

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Curriculum vitae

The author K. Ganesh Kumar was born on 17th May, 1973 in Tirupur, Tamilnadu, India. Did his school studies in Tirupur. Completed his B.V.Sc in Veterinary college and research institute, Namakkal, Tamilnadu Veterinary and animal sciences university on 1996. With the help of ICAR-JRF he completed his M.V.Sc degree in Animal Genetics from Indian Veterinary Research Institute on 1998. Continued his Ph.D in IVRI, meanwhile got selected for DAAD sandwich model fellowship and did his research in University of Bonn, Germany (2001 – 2003) for his thesis work.