PRODUCTION AND CHARACTERIZATION OF ANTI-IDIOTYPIC ANTIBODIES FOR THE CONTROL OF *ESCHERICHIA COLI* INFECTIONS IN MAMMALS

A thesis

Submitted to the Faculty of Graduate Studies

The University of Manitoba

By

Ziad Waheed Jaradat

In Partial Fulfilment of the Requirements for the Degree

of

Doctor of Philosophy

In

Food and Nutritional Sciences

September 1999

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of DOCTOR OF PHILOSOPHY

Abstract

Enterotoxigenic *Escherichia coli* (ETEC) cause diarrheal diseases in both humans and domestic animals. It is a prominent problem in swine industry and it is responsible for high mortality in neonatal piglets. Therapeutic antibodies offer an alternative approach to the use of antibiotics for solving these problems. An experimental program was undertaken to evaluate the efficacy of therapeutic antibodies in both mice and neonatal piglets. Polyclonal anti-idiotypic antibodies (anti-antibody, for short pAb2) were produced in laying hens against anti-fimbrial antibodies obtained from mice (mouse monoclonal antibodies, mAb1) and rabbits (rabbit polyclonal antibodies, pAb1). The chicken serum was purified and tested for the presence of Ab2 that mimic the K88 fimbrial antigen using the indirect competitive ELISA. The pAb2 that was produced against anti-K88 pAb1 inhibited the binding of K88 fimbriae to pAb1 by 30-35 % while pAb2 produced against mAb1 inhibited this binding by only 6-15 % depending on the type of mAb1 that was used for immunizing the chickens. Pure pAb2 were injected into mice to produce pAb3 with anti-K88 activity. As determined by ELISA, higher pAb3 values were obtained from mice immunized with the pAb2 that were produced against pAb1s compared to those produced against mAb1s. The survival rates in mice that were immunized with six different Ab2 and then challenged with an IP injection of *E. coli* K88 were 100 % in the two experiments, while the corresponding survival rates in the *E. coli* treated, non-immunized groups was only 25 and 18 % . A second study was undertaken to evaluate efficacy of two pAb2 preparations produced against rabbit IgG2a and its Fab2 fragments as therapeutic agents for neonatal diarrhea in piglets infected with *E. coli* K88. Unlike the mice protection experiment, treatment of piglets with oral doses of pAb2 yielded only partial protection (50 % survival rate) among the challenged piglets, while only 13.5 % of control group survived.

In addition, anti-idiotypic antibodies were used for *E. coli* K88 receptor identification in porcine intestinal mucus. *E. coli* K88 receptors were purified from porcine intestinal mucus by affinity chromatography using a K88 fimbrial antigen attached to Sepharose 4B. The isolated receptors were then identified using anti-idiotypic antibodies in conjunction with electrophoresis and immunoblotting. Four major proteins corresponding to 40, 45, 50 and 70 kDa were identified when probed with K88 fimbriae. However, when probed with the pAb2 preparations, only the70 kDa protein was recognized by pAb2 produced against rabbit pAb1, while pAb2 produced against mAb1 failed to recognize any of these proteins. Sugar staining suggested that only the 45 kDa protein contained a sugar moiety.

The effect of non-reducing sugars (sucrose, lactose and trehalose), complex carbohydrates (cyclodextrin and dextran), infant formula and egg-yolk on the stability of chicken IgY was also studied under different conditions. Regardless of the protectant that was used, about 20 % of IgY activity was lost during freeze-drying except in case of infant formula where a bout 75 % of IgY activity was lost. IgY activity was completely lost after pepsin treatment except when treated in the presence of infant formula and egg-yolk where 34 and 40 % of its activity was retained, respectively. IgY was fairly stable after trypsin treatment with the recovery of activity being between 75- 100 % depending on the protectant. IgY, when heated in aqueous solutions containing the different protectants, was found to be stable at 50, 60 and 70 °C, however, complete loss of its activity was observed at 80 and 90 °C. The exception was infant formula and egg-yolk where about 5 % of the IgY activity was still recovered. These studies have indicated that therapeutic antibodies can be successfully utilized to control infections in humans and animals. In addition, they can be used as diagnostic agents for receptor identification. Specific chicken IgY can be added as a supplement to

the human diet particularly infant formula as they are able to resist certain processing and digestive conditions.

Acknowledgments

I would like to thank Dr. R. R. Marquardt, (thesis advisor) for his excellent support, advise and guidance throughout my PhD program. Many thanks are extended to Dr. A. A. Frohlich for his invaluable assistance, to Dr. Gregory Blank for his continuous support and especially with the microbiological studies and to Dr. S. K. Baidoo and Dr. Arun Bhunia (External examiner/ Purdue University) for reviewing this thesis.

The technical assistance of Chokri Zghal, Jim Rogers, Harry Muc, Shirley McFaren and Douglas Durnin is greatly appreciated.

I greatly acknowledge the Department of Animal Science for providing their facilities. The financial support provided by the University of Manitoba Graduate Fellowship, the Natural Sciences and Engineering Council of Canada (NSERC), Agriculture and Agri-Food Canada and Manitoba Agriculture is acknowledged.

Finally, my sincere thanks and appreciation are extended to all members of my family, for their endless support and for my wife, Rasha, for her support and patience.

Forward

This thesis has been written in the manuscript format. Although these manuscripts are interrelated for the most part, each represents an independent study and has been prepared according to the instruction of the journal that it was submitted for or will be submitted to. Here are the authors and titles of these manuscripts:

1· Jaradat, Z. W., Marquardt, R. R. and Frohlich, A. A. 1999. Use of chicken anti-idiotypic antibodies for the treatment of diarrhea caused by *Escherichia coli* K88 infection. (Submitted to Journal of Immunolgy).

2· Jaradat, Z. W. and Marquardt, R. R. 1999. Identification of *E. coli* K88 Receptor in Porcine Intestinal Mucus Using Anit-idiotypic antibodies. (Submitted to Journal of Immunolgoical methods).

3· Jaradat, Z. W. and Marquardt, R. R. 1999. Studies on the stability of Chicken IgY in Different Sugars, Complex Carbohydrates and Food Materials.(Submitted to Food and Agricultural Immunolgy).

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ab1	**Antibody 1**
Ab2	**Anti-idiotypic antibodies**
BCA	**Bicinchoninic acid**
BCIP	**5-bromo-4-chloro-3-indolyl phosphate**
CBB	**Coomassie brilliant blue**
Cyclodextrin	**2-Hydroxypropyl-β-cyclodextrin**
CFA	**Complete Freunds Adjuvants**
CFU	**Colony forming unit**
ELISA	**Enzyme linked immunosorbent assay**
EHEC	**Enterohemorrhagic *Escherichia coli***
EIEC	**Enteroinvasive *Escherichia coli***
EPEC	**Enteropathogenic *Escherichia coli***
ETEC	**Entero toxigenic *Escherichia coli***
FPLC	**Fast protein liquid chromatography**
HH buffer	**Hepes-Hanks buffer**
IFA	**Incomplete Freunds Adjuvants**
IP	**Intraperitoneal**
kDa	**Kilodalton**
LPS	**Lipopolysaccharide**
mAb	**Monoclonal antibody**
MW	**Molecular weight**

NBT	Nitro blue tetrazolium
NC	Nitrocellulose
OD	Optical density
pAb	Polyclonal antibody
PBS	Phosphate buffered saline
PBST	PBS with Tween 20
pNPP	p-Nitrophenyl phosphate
PWD	Post weaning diarrhea
RBP	Retinol binding protein
SDS-PAGE	Sodium dodeycl sulfate polyacrylamide gel electrophroesis
T-2	Trichothecene mycotoxin
TBS	Tris buffered saline
TBST	TBS with Tween 20
TSB	Tryptic soy broth

1. Introduction

Escherichia coli are inhabitants of the intestinal tract of humans and animals (Doyle and Clever, 1990). They colonize the intestine shortly after birth and eventually constitute the major portion of intestinal flora (Gyles, 1993). Generally, *E. coli* strains that colonize the intestine are harmless with the exception of few pathogenic strains that causes diarrhea and other gastrointestinal diseases in both humans and animals (Padhye and Doyle, 1992). Enterotoxigenic *Escherichia coli* (ETEC) strains bearing the K88 fimbrial antigens are considered the most frequent causative agents of enteric colibacillosis encountered in neonatal and early weaned piglets (Yokoyama et al, 1992; Marquardt et al, 1999). Among the major causes of diarrhea in piglets are ETEC strains bearing K88, K99 and 987P fimbriae with *E. coli* K88 being the most prevalent form (Laux et al, 1986; Metcalf et al, 1991; Erickson et al, 1992; Blomberg et al, 1993; Marquardt et al, 1999). The absence of strong gastric acids, absence of established intestinal flora and the presence of specific receptors in the small intestine are all factors contributing to the development of diarrhea in neonatal piglets (Smith et al, 1992).

The first and most critical step in initiating the infection by many pathogens is the recognition of specific host cell receptors and the consequent attachment of pathogens to these receptors (Thaker et al, 1994). The attachment of *E. coli* through its fimbriae to intestinal receptors allows it to resist and circumvent cleansing and removal by intestinal peristalsis, therefore enabling it to colonize the intestine leading to infection by release of enterotoxins (Klemm, 1985; Linton and Hinton, 1988; Erickson, 1992, 1997; Nagy et al, 1992; Nystrom, 1995).

During the past few decades, antibiotics have been the therapy of choice for the control and prevention of animal diseases. However, their routine use has contributed widely to the appearance

of multiple drug resistant *E. coli* and other pathogens thereby limiting their use (Hay, 1986).

Vaccinating mothers with particular antigen such as K88 fimbriae leads to the production of specific antibodies that pass on to the neonate through colostrum and milk. This approach was used to control some prominent animal diseases but due to the low amount of antibodies that pass through milk and the short period of colostrum secretion it is not practical on a large scale (Isaacson, 1994).

Carbohydrate or lipid receptors that are found on the surface of epithelial cells form the basis of bacterial attachment. Neeser et al, (1986) reported the blocking of this attachment by feeding piglets with excess amounts of mannoside oligosaccharides obtained from kidney beans thus, preventing infection. However, large amounts of such carbohydrates were needed to prevent the infection rendering the approach impractical.

An alternative approach that seems appealing is the use of therapeutic antibodies to confer passive immunity. Oral administration of antibodies with pre-defined specificity obtained from colostrum (Shimizu et al, 1988), serum (Spier et al, 1989) or monoclonal antibodies (Sherman, 1983; Smith and Lida, 1990) have been used to combat intestinal diseases both in humans and animals. Unfortunately, it is prohibitively expensive or impractical to obtain large amounts of these antibodies (Yolken et al, 1988).

Egg-yolk is a rich source of antibodies that are readily extracted and purified (Jensenius et al, 1981; Grossman et al, 1990). It is possible to obtain antigen specific IgY by immunizing chicken with the desired antigen (Bartz et al, 1980; Yolken et al, 1988; Otake et al, 1991; Yokoyama et al, 1992). Chicken IgY has been successfully used for therapy of rotavirus infection in mice (Bartz et al, 1988; Yolken et al, 1988), neonatal diarrhea caused by *E. coli* K88, K99 or 987P in piglets (Ikemori et al, 1992; Yokoyama et al, 1992), *E. coli* F18 infection in weaned piglets (Zuniga et al,

1997) or for the prevention of dental carries caused by *Streptococcus mutants* (Otake et al, 1991). Current approaches to produce these therapeutic antibodies are mainly though immunizing a host with an attenuated or inactivated pathogen or just part of its surface antigens. However, certain pathogens cannot be grown in vitro such as viruses ,also some organisms are extremely dangerous and difficult to grow such as *Mycobacterium liperae* or *Legionella* or they are extremely hazardous (Dreesman and Kennedy, 1985; Ertl and Bona, 1988)

Studies have provided evidence that anti-idiotypic antibodies (Ab2) that mimic an antigen located on the surface of a pathogen can be produced by immunizing a host with antibodies (Ab1) that recognize this particular antigen (Jerne, 1974; Rico and Hall, 1989). The Ab2 that mimic the antigen can induce an immune response against that antigen in individuals or animals that have never encountered this pathogen (Ertl and Bona, 1988; Rico and Hall, 1989). Therefore this minimizes the need for extensive and lengthy dealing with hazardous antigens. Anti-idiotypic antibodies have been used to induce protective immunity to several pathogens including influenza, blue tongue, bovine herpes, hepatitis viruses (Kennedy et al, 1984, 1986; Anders et al, 1989; Grieder and Schulttz, 1990; Srikumaran et al, 1990); and *Clostridium chauvoei*, *Clostridium perfringens*, *Listeria monocytogenes, Chlamydia trachomatis, Streptococcus pneumonia, E. coli* K13 and *Brucella abortus* (McNamara et al, 1985; Stein and Soderstrom, 1984; Kaufmann et al, 1985; Beauclair and Khansari, 1990; Percival et al, 1990; Brossay, 1994;Tanaka et al, 1994). In addition, anti-idiotypic antibodies have been reported to induce partial protective immunity against parasites such as *Trypanosoma* and *Schistosoma* (Crzych et al, 1985; Sacks et al, 1982). Furthermore, anti-idiotypic antibodies have been used as therapeutic agents in which they were administered orally and provided partial protection against emetic response and diarrhea in monkeys (Reck et al, 1988). They

also have provided partial protection against dental carries in rats (Jackson et al, 1990). However, other than these studies, there have been no investigations on the use of anti-idiotypic antibodies as therapeutic agents against gastrointestinal diseases in humans or animals.

In addition to the use of anti-idiotypic antibodies as therapeutic and prophylactic agents, they have proven to be exceptional tools for the identification of cell surface receptors (Gaulton et al, 1984). Their use for receptor identification was first reported for the retinol binding protein and insulin by Sege and Peterson, (1978). Following this report, several other receptors were identified using their methodology. These reports include the identification of receptors for reovirus (Noseworthy, et al, 1983), atrial natriuretic factor (Chua et al, 1989), bovine herpes virus 1 (Thaker et al, 1994) and many other receptors. However, at present no reports exist on the identification of receptors for gastrointestinal pathogens using anti-idiotypic antibodies.

Chicken IgY antibodies, due to their availability and ease of production and purification, would seem to be excellent reagents either for therapy or receptor identification. Therefore, their stability is very important since therapeutic use would expose them to digestion and denaturation while passing through the stomach. Several studies have been conducted to evaluate their stability. Shimizu et al, (1988, 1992, 1993b, 1994) and Otani et al, (1991) studied the stability of IgY under heat, acid treatment as well as enzyme digestion. They found that chicken IgY is fairly stable under these conditions. However, the IgY was studied in PBS buffer without that addition of protectants except in one study conducted by Shimizu et al, (1994) were they evaluated the stability of IgY in the presence of 30-50 % sucrose.

Therefore the objectives of this thesis were: i) to produce chicken anti-idiotypic antibodies that bear an internal image of *E. coli* K88 fimbrial antigen and evaluate their ability to block *E. coli*

K88 binding to intestinal receptors thus preventing diarrhea in experimentally challenged piglets , ii) to evaluate their efficacy in inducing protective immunity when injected in a mouse host and subsequently, challenged with *E. coli* K88, iii) to use the anti-idiotypic antibodies for the identification of *E. coli* K88 receptor(s) in porcine intestinal mucus, and iv) to evaluate the stability of the chicken IgY in the presence of infant formula, egg-yolk, sugars and complex carbohydrates under certain denaturing conditions that occur naturally during processing or when IgY is exposed to conditions similar to those that occur in the intestine.

2. Literature Review

2.1 Escherichia coli

Escherichia coli are inhabitants of the intestinal tract of humans and animals. They are non-spore forming gram negative rods, with a size of about 0.5 to 1.0 μm (Doyle and Cliver, 1990; Weeratna and Doyle, 1991). Generally they are motile, catalase positive, lactose fermenters, and have an optimum growth temperature of between 30 and 37 °C (Doyle and Cliver, 1990; Weeratna and Doyle, 1991). *E. coli*, was first recognized by Theodore Escherich in 1885 (Gyles , 1993). The name of *E. coli* was derived from it's discoverer's name and it's principal habitat, the colon (Gyles, 1993).

Several methods have been developed to characterize *E. coli*. These methods include serotyping, phage typing, biotyping, electrophoretic typing, virotyping (testing for virulence factors) (Gyles, 1993) and DNA profiling (genomic fingerprinting). Nevertheless, serotyping which is based on different antigens, such as O (somatic), K (capsular) and H (flagellar) is the most widely used (Ewing, 1986). In addition *E. coli* is also classified according to fimbrial antigens which are small appendages produced by the organism if grown under optimal conditions (De Graaf, 1988). Several studies have indicated that the biosynthesis of different types of fimbriae are affected by the specific rate of growth of the organism, the incubation temperature and the composition of the growth medium (De Graaf, 1988).

E. coli colonize the intestine of humans and animals shortly after birth and eventually constitute the major portion of the intestinal flora (Gyles, 1993). Generally, the *E. coli* strains that colonize the intestine are harmless with the exception of a few strains that are pathogenic. These strains cause different degrees of diarrhea and other related diseases in both animals and humans

(Padhye and Doyle, 1992).

2.1.1 Classification of Escherichia coli

E. coli that are associated with foodborne illness are categorized into four different types based on virulence, clinical syndromes, toxin production and distinct O:H:K antigens (Padhye and Doyle, 1992). These groups are ; enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC); enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC). A disease in pigs that is caused by the latter type of *E. coli* will be the focus of this thesis (Kornacki and Marth, 1982; Levine, 1987; Olsvik et al, 1990; Pollard et al, 1990; Smith, 1992; Padhye and Doyle, 1992).

2.2 Fimbriae

2.2.1 Definition: Fimbriae or pili are thread-like structure projecting from the bacterial surface of practically all gram negative and some gram positive bacteria (Smyth et al, 1996; Mol and Oudega, 1996). They have a polymeric protein structure (Mol and Oudega, 1996). These non-flagellar , filamentous appendages were originally recognized by Houwink and van Iterson in 1950 (Smyth et al, 1996). These fimbriae or pili possess adhesive properties by which it adheres to specific receptors at mucosal surfaces or other body tissues and consequently are partially responsible for the disease (Mol and Oudega, 1996; Smyth et al, 1996). The bacteria by adhering to surfaces, use these appendages to resist and circumvent the host defense mechanisms. Such mechanisms are urination, desquamation and peristaltic propulsion in addition to normal cleansing mechanisms that protects the epithelial and mucosal surfaces in higher animals (Klemm, 1985; Smyth et al, 1994).

The word fimbriae came from the Latin word for thread, while the term pili came from the

Latin word for hair-like structure (Mol and Oudega, 1996). Although both terms are used interchangeably, the term fimbriae is more commonly used for adhesive properties, while pili is a term used to denote sexual appendages (Klemm, 1985; Mol and Oudega, 1996).

*2.2.2 **Structure***: Most fimbriae when examined by the electron microscope appear as a rod-like structure having a diameter of 2-7 nm and a length of up to 2μm. This includes fimbriae such as type 1 fimbriae, 987P, and CFA I (Klemm, 1985; Bakker, 1991a; Hacker, 1992; Smyth et al, 1994). Some fimbriae such as K88, and K99 appear thinner and wiry with a diameter of about 2-4 nm (Smyth et al, 1996; Smyth et al, 1994). Although the different types of *E. coli* fimbriae differ in morphology, they all are peritrichously distributed on the bacterium in numbers of from 100-1000/cell, with the protein subunits being about 1000 per fimbriae (Klemm, 1985; Hacker, 1992). *E. coli* fimbrial adhesions are composed mainly of a major subunit which consists of identical repeating protein subunits and minor fimbrial subunit (Bakker, 1991a; Smyth et al, 1994).

*2.2.3 **Classification***: According to the appearance of fimbriae under an electron microscope, they can be grouped into two main groups: i) the first group consists of fimbriae with subunits arranged in a tightly packed right handed helix having a diameter of 7 nm ii) ,while the second group consists of thin round more flexible structures with a poorly defined diameter of about 2-4 nm (Mol and Oudega, 1996).

The other method of classifying fimbriae is based on their adhesive properties. It has been shown that in addition to their receptors, fimbrial adhesions bind to the red blood cells of various animals (Mol and Oudega, 1996; Smyth et al, 1996). This property is utilized in hemagglutination

assays to classify fimbriae into two categories according to their sensitivity to inhibition by D-mannose: mannose-sensitve and mannose-resistant fimbriae (Mol and Oudega, 1996). However, this classification cannot be used for all bacteria since some fimbriae such as 987P do not mediate hemagglutination (Mol and Oudega, 1996). Since all our studies were focused on *E. coli* bearing K88 antigen, only this type will be discussed further.

2.3 K88 Fimbriae

The K88 antigen was first described by Orskov et al, (1961). It was first recognized on the surface of porcine enterotoxigenic *E. coli* (Bakker, 1991b; Mol and Oudega, 1996). Further, the K88 fimbriae was the first to be discovered among those mannose resistant pili. It was first given a K antigen number, because it fit neither the O nor H antigenic formula. Recently, it was reclassified and was designated as K88 (Gyles, 1993). This fimbrial antigen was found to mediate the adhesion of the bacterial cell to the small intestine of new born piglets. This adherence enables this strain of *E. coli* to colonize and attain high population density in the gut and to eventually cause diarrhea (Klemm, 1985). K88 fimbriae is composed of a single repeating protein subunit called FaeG. It consists of 264 amino acid residues and has a molecular weight of 27,500 Daltons (Klemm, 1985; Bakker, 1991b, 1992) and is responsible for the hemagglutination of different animal enterocytes (Bakker, 1991b, 1992). Furthermore, the major subunit of K88 possess adhesive properties that enable bacteria to attach to the mucosal surfaces (Bakker, 1991b). In addition to FaeG, there is a minor fimbrial subunit designated FaeC. It has been detected only in low amounts and was found to possess limited adhesive properties (Bakker, 1991b; Smyth et al, 1994).

K88 antigens are most frequently associated with O serogroups 8, 45, 138, 141, 147, 149, and

157 (Gyles, 1993). In addition K88 fimbriae exists in four immunologically distinct antigenic types; K88ab, K88ac, K88ad, and K88ad(e), (Klemm, 1985; De Graaf, 1988; Bakker, 1991b; Gyles, 1993; Mol and Oudega, 1996; Mynott et al, 1996). The differences between these serotypes of K88 fimbriae are found in the major subunit (FaeG), (Mol and Oudega, 1996). These different antigens have a common antigenic factor termed a, whereas b, c, d, and d(e) are variable factors (Klemm, 1985).

When these variants were studied using monoclonal antibodies raised against all K88 variants, it was revealed that at least five different epitopes exist which are common in two or all of them. However, each of them was found to have one type specific epitope (De Graaf, 1988). This variance is not likely to be due to immunological pressure imposed on bacteria by the host, instead, it could be due to further specialization on particular hosts carrying different receptors (Mol and Oudega, 1996). The differences in hemagglutination patterns by different K88 variants supports the above suggestion (Mol and Oudega, 1996).

K88 fimbriae are produced at 37° C (normal body temperature) but not at room temperature (18-21° C), (Gyles, 1993).

2.3.1 Isolation of K88 fimbriae

Several methods have been used to prepare pure K88 fimbriae from bacterial cell homogenates. Sonication, homogenization and heat extraction with or without detergents is the major method most commonly used for fimbrial extraction (Bakker et al, 1992; Payne et al, 1991). Payne et al, (1991), used 2 M urea instead of a detergent along with heating of the bacterial suspension for 20 min at 60° C. The suspension was centrifuged and further precipitation of the

fimbrial antigen was performed using 60% ammonium sulphate. Bijlsma et al, (1982) grew bacteria on diagnostic sensitivity test agar, after harvesting and shearing fimbriae by homogenization for 30 min on ice, the cells were centrifuged and pili in the supernatant were precipitated with either 60% ammonium sulphate or by ultracentrifugation (Jacobs and De Graaf, 1985). Erickson et al, (1992) used a combination of both procedures, heating the bacterial suspension for 30 min at 60°C followed by homogenization of the suspension for 10 min to shear the fimbrial antigen from the cells. Repeated isoelectric precipitation was used for the purification of the pili from the supernatant. The latter method was used routinely in our laboratory.

2.4 Fimbrial intestinal receptors

The ability of ETEC to colonize the small intestine is an important virulence factor as it is a prerequisite for the production of diarrhea in both humans and animals (Erickson et al, 1992; Nystrom, 1995). This colonization, as indicated previously is facilitated by the binding of bacterial adhesions called fimbriae or pili to specific receptors on the villus epithelium (Erickson et al, 1992; Nystrom, 1995). The susceptibility of pigs to *E. coli* K88 infection is age dependent with susceptibility being high in neonatal and postweaning piglets and less at 35 days and older (Willemsen, 1993)

Two different phenotypes of porcine intestinal brush border occur in nature, adhesive and non-adhesive. The difference between both is determined genetically with the adhesive gene dominant over that of the non-adhesive and inheritance occurring in a simple Mendelian manner (Kearns and Gibbons, 1979; Erickson et al, 1992). There are five phenotypes of pigs that are distinguished by the K88 adhesion variants (K88ab, K88ac and K88ad) that bind to their brush

border epithelial layer (Billy et al, 1998). These phenotypes are :(A) all three variants,(B) K88ab and K88ac,(C) K88ab and K88ad, (D) K88ad and (E) none of the variants. The first four phenotypes belongs to the adhesive type while the fifth phenotype is designated as non-adhesive (Billy et al, 1998). Furthermore, it was found that once the bacterium adheres to the epithelium it colonizes and eventually cause the disease. Therefore, the adherence of the bacteria to mucus receptors might be an initial defense mechanism by preventing their penetration to the epithelium (Willemsen, 1993).

2.4.1 Nature of receptors

The exact biological nature of K88 adhesion-receptors has not been fully elucidated (Metcalfe et al, 1991). However, it appears from the literature that these receptors might either be glycoproteins or glycolipids (Laux et al,1986; Erickson et al, 1992; Blomberg et al, 1993; Smith et al, 1994; Nystrom, 1995). Results of previous studies in which sugars were used to block the adhesion of K88 to brush border cells or the mucus of pig small intestine suggested that galactosyl, N-acetylglucosamine, N-acetylgalactosamine and D-galactosamine might be constituents of the putative receptors for K88. (Laux et al, 1986; Metcalfe et al, 1991; Smith et al, 1994). Smith et al, (1994) suggested the following chemical structure for the K88 glycoprotein receptor; Gal(β1-3)GalNAc or Fuc(α1-2) Gal(β1-3/4)GlcNAc.

2.4.2 Receptor identification for E. coli K88 Pili

Early reports (Kearns and Gibbons, 1979) stated that the identity of the K88 receptor is a hydrophilic glycolipid and posses oligosaccharide chains composed of 4-6 sugars but no molecular weight was determined. Staley and Wilson, (1983) and Wilson et al, (1984) have identified two

small molecular weight receptors for K88 fimbriae. These receptors have a MW of 23 and 32 kDa and were identified in porcine brush borders. Laux et al, (1986) have identified and characterized mouse mucosal receptors that bind to *E. coli* K88ab. Three more receptors were identified; the 57 and 64 kDa receptors were isolated from both the brush borders and the mucus, while the third, 91 kDa receptor was found exclusively in the brush borders. All of these receptors were characterized as glycoproteins which may contain D-galacotsamine or D-galactosamine-like residues. Several laboratories were subsequently involved in the identification and characterization of receptors from pig intestine. Metcalf et al, (1991) used gel filtration and electrophoresis to identify a receptor that is found in the mucus of the porcine small intestine. The receptor had a MW of 40-42 kDa and was a glycoprotein. Erickson et al, (1992) used preparative gel electrophoresis to identify, excise and elute the putative K88ac receptors from porcine brush borders. Two receptors with MW of 210 and 240 kDa were identified. Further, the authors proved that these receptors are present only in the adhesive brush borders. In a second study, Erickson et al, (1994) characterized the identified brush border receptors. They reported that both receptors were mucin type sialoglycoproteins containing O-linked oligosaccharides. Further, they have concluded that both receptors may be closely related and possibly are variants of the same glycoprotein. Willemsen, (1993) reported that porcine intestinal mucus contained 25, 35 and 60 kDa proteins that bound strongly to both K88ab and ac but weakly to K88ad. In addition, brush borders were found to contain several proteins ranging in MW from 40-70 kDa and a small MW protein of 16 kDa that also bound K88 fimbriae. Studies with the blocking of the adherence of receptors to K88 fimbriae using several carbohydrates confirmed the carbohydrate nature of the mucus receptors, however, no lectin inhibition was observed with brush border proteins.

Studies were also designed to detect the presence of glycolipid anti-K88 receptors. Blomberg et al, (1993) reported that there was glycolipid receptor in the piglet small intestine mucus. The receptor appeared to be a galactosylceramide which was detected in both the total and neutral lipids. The adhesion of the putative glycolipid receptor to K88 fimbriae was inhibited completely by crude K88 fimbriae, thus, confirming that it served as a receptor for K88 adhesion.

In a recent study, Erickson et al, (1997) proposed a three receptor model for the *E. coli* K88 that accounts for the several phenotypes; i) receptor b,c,d, binds all variants ii) receptor bc binds K88 ab and K88ac and iii) receptor d binds K88ad. They further characterized the previously identified 210 and 240 kDa receptors, which belong to the bc category.

2.5 Diarrhea in piglets

2.5.1 Neonatal diarrhea in piglets: Diarrhea is one of the most prevalent diseases in neonatal piglets worldwide (Yokoyama et al, 1992). The diarrhea caused by ETEC is caused by the ingestion of the bacteria from the surroundings. The absence of strong gastric acids and established intestinal flora and the presence of specific receptors for ETEC in the intestine contribute positively to the development of diarrhea in neonatal piglets (Smith, 1992). In a survey of pre-weaning diseases in the UK, diarrhea represented approximately 11% of pre-weaning mortality (Alexander, 1994). Piglet diarrhea may occur at any time during suckling. However, the highest incidence occurs in the first 3-5 days of age. ETEC is considered the sole source of these infections, while other agents might cause diarrhea in older pigs (Alexander, 1994).

ETEC strains do not invade epithelial cells of the intestine, instead they attach to the brush borders of the enterocytes on the villi where they releases their enterotoxins (Linton and Hinton,

1988; Nagy et al, 1992a). The toxin release leads to a condition resembling cholera in man. The presence of large numbers of *E. coli* in the small intestine is accompanied by excessive loss of fluids and electrolytes (Linton and Hinton, 1988).

2.5.2 Post-weaning diarrhea: It is not uncommon for piglets to develop diarrhea 3 to 10 days after weaning (Hampson, 1994). Most pig farmers now tend to wean their piglets at 3-4 weeks of age. This trend toward early weaning may be implicated in this post-weaning diarrhea (PWD), (Hampson, 1994). Several factors may lead to PWD ; introduction of the use of a dry-plant based diet that has a high content of complex carbohydrates, and consumption of soy bean proteins which may not be readily digested, also, piglets may be hypersensitive to soy bean proteins (Gyles, 1993). It has been suggested that immune-mediated intestinal damage occurs in early weaned piglets when they are introduced to solid food at the time of weaning (Gyles, 1993). Further, the loss of sow's milk with it's antimicrobial properties (transferrin, lactoferrin, antibodies and lectins that bind fimbrial adhesions) contribute to the PWD (Gyles, 1993). Other factors including the development of antibiotic resistant ETEC by the use of antibiotics in feed and decreased gastric activity by a temporary increase in stomach pH may contribute to PWD (Sarmiento et al, 1988).

Hemolytic *E. coli* particularly serogroups O:8, O:138, O:141, O149, O:157 and K88ac have also been incriminated in PWD with serogroup O:149 being the most frequent (Linton and Hinton, 1988 ; Gyles, 1993).

Similar to neonatal diarrhea, enterotoxins released by colonizing ETEC are responsible for the clinical symptoms (Cassels and Wolf, 1995). However, the mortality in post-weaning appears to be much lower than that in neonatal diarrhea (Linton and Hinton, 1988).

2.6 Conventional therapeutic approaches

2.6.1 Antibiotic treatments: During the past few decades antibiotics have been the therapy of choice for the control of animal diseases. Their wide-spread use had a recognizable and profound impact on the life and health of humans and animals. Sub-therapeutic doses are widely used for treatment and prevention of diseases in animals under stress and as growth promoters in livestock and poultry production. Antibiotics are also used in the control of wildlife, fish, plant diseases and food spoilage (D'Aoust et al,1992). Approximately, 80 % of poultry, 75% of swine, 60% of beef cattle and 75 % of dairy calves marketed or raised in the USA have been estimated to receive antibiotics at some point in their life (Hays, 1986).

2.6.1.1 Drawbacks of antibiotic use in animal industry: In the last two decades concern has repeatedly been expressed by health scientists, that using antibiotics on a routine basis at a sub-therapeutic level in animal feed and their frequent uses as therapeutic agents has contributed widely to the appearance of multiple drug resistance in bacteria such as *Salmonella* and *E. coli* (Hays , 1986).

The issues of multiple drug resistant bacteria came to the fore-front in 1965 in England when there was an epidemic of drug resistant *Salmonella typhimurium* in dairy calves that subsequently spread to the humans. Thousands of animals and seven people died as a result of the epidemic which lasted several years. The bacteria were found to be resistant to eight different antibiotics (Black, 1984; Dupont and Steel, 1987). Of particular concern is not only the appearance of organisms that have multiple resistance to several antibiotics but their ability to elaborate new toxins (Marquardt, 1997). The recent outbreaks of *E. coli* O157:H7 infections in humans demonstrated the

ability of *E. coli* to rapidly adapt to new hosts, new conditions and subsequently develop resistance to new antibiotics (Marquardt, 1997). Furthermore, the ingestion of low levels of antibiotic residues in meat products imposes a potential hazard of inducing allergies in sensitized humans. Finally, the detection of antibiotic residues in meat products violates health regulations and subjects the producer to fines and subsequently condemnation of dairy, poultry and meat products. In order to give a full coverage of the potential hazards of extensive antibiotic use, it is important to review the mechanism by which drug resistance is developed in bacteria and its effect on public health.

2.6.1.2 Plasmid mediated antimicrobial drug resistance

Plasmids are extrachromosomal, double stranded, and covalently closed circular DNA molecules found in bacterial cytoplasm (Lancini and Parenti, 1982). Resistance to antimicrobial drugs is commonly encoded in large plasmids or what is called the R factor. This R factor is widely distributed in bacteria such as *E. coli, Shigella, Vibrio* and others. It is composed of 2 parts : i) a resistance transfer factor which is responsible for replication and transmission and ii) resistance determinants. The resistance factor functions in four different ways (Lacini and Parenti, 1982):

1) encoding of drug inactivating enzymes; e.g, β-lactamase against β- lactam antibiotics,

2) decrease bacterial cell permeability to antibiotics,

3) decrease affinity of the drug to cell components such as ribosomes, and

4) increase the production of components inhibited by the antibiotic.

Multiple resistance is normally acquired by gene modification that encodes for an expanded substrate profile (i.e, more than one antibiotic) by insertion into the plasmid of one or more transposons. These transposons are highly mobile DNA fragments which move from one location

to another within a plasmid, from one plasmid to another or between plasmid and chromosomal locations. The end result is that bacteria may acquire instant and transferable resistance to various combinations of antibiotics and sulfonamides (Steele and Berna, 1984).

2.6.1.3 Antibiotics resistance and public health

The wide-spread use of antimicrobial agents in animal feed has been a major concern in human medicine with special consideration on: indirect effects on the acquisition of antimicrobial resistance by bacterial strains affecting humans and the possible effects of increasing the reservoir of non-typhoid *Salmonella* in humans (Frappoalo, 1983 and Hampson, 1994 and).

Several ways in which the routine use of antimicrobial agents in animal feeds may impose potential hazards to humans are:

i) pathogenic organisms such as *Salmonella* existing in the gastrointestinal tract of animals may acquire resistance to antibiotics which may then be passed on to the human food chain,

ii) resistance could be developed in non-pathogenic bacteria, such as *E. coli,* which may be transferred to human pathogens, and

iii) there is also a concern that frequent use of antibiotics in animal treatment and feed may increase the prevalence or prolong the shedding of *Salmonella* organisms, thus , increasing the likelihood of cross-contamination from animals to humans and from humans to humans (Frappoalo, 1986).

These observations suggest that antibiotic use in the livestock and poultry industry should be limited and subjected to more severe regulation. Many antibiotics are now being banned for use in livestock particularly in Europe. It is therefore urgent that alternative strategies be developed to control intestinal pathogens.

2.6.2 Vaccination

It is not possible for the undeveloped immune system of piglets to develop active immunity as neonatal diarrhea occurs at an early stage. Therefore, passive immunity might hold the solution for such a problem (Isaacson, 1994). Vaccinating the mothers with a particular antigen, allows their immune system to develop specific antibodies to that antigen. Upon lactation, the antibodies will pass through colostrum and to a lesser degree normal milk to their suckling piglets.

Two general approaches are mostly used for such vaccines. The empirical, where live or killed whole bacterial cells are given to the mothers before parturition. In turn, mothers will elicit a battery of antibodies against the several antigenic determinants on the surface of bacteria, thus, conferring protective immunity in the suckled piglets. The other main approach utilizes virulence factors of the said bacteria, such as fimbrial adhesions and enterotoxins (Isaacson, 1994). Early attempts utilizing the aforementioned approaches goes back to the late 1950's .

Gordon and Luck, (1958) reported that vaccination of sows with whole *E. coli* cells reduced diarrhea and mortality of piglets, while Jones et al, (1962) reported that vaccination of sows had no apparent effect on the incidence of diarrhea. Rutter and Anderson, (1971) reported that vaccination of dams against K88ac before parturition reduced the mortality of piglets challenged with *E. coli* from 38 to 20 % although the reduction in mortality was not significant when the variation between litters was taken into account. The course of diarrhea was shorter in the piglets of vaccinated sows. Morgan et al, (1978) reported that suckling piglets of sows that were vaccinated parenterally with *E. coli* K99 fimbriae were protected against fatal diarrhea upon challenge. Even, when live ETEC organisms were fed for three days to pregnant sows, their suckling piglets were protected upon subsequent challenge with the same strain as that which was fed to the mothers

(Kohler et al, 1975, 1978). Moon, (1981) proved that the protective antigen was the pilus. Sows vaccinated orally with live ETEC produced colostral antibodies against pili and as a result, suckling piglets were more resistant to ETEC challenge than the control piglets. Nagy et al, (1985) reported that inclusion of defined quantities from each of K88ab and ac, K99 and 987P firmbrial antigens resulted in a highly effective vaccine. The vaccine conferred protection for the litters of the vaccinated dams against the bacteria. Further, the duration of diarrhea among vaccinated litters was reduced significantly and the excretion of K88 was reduced compared to that of K99 and 987P bacteria.

It has been suggested by some researchers that immunization is not an absolutely safe method of protection and that extensive use of vaccines will rapidly select against prevailing fimbrial antigen types leading to the emergence of new, prevalent fimbrial antigens. (Moon and Bunn, 1993). This later possibility however, is not likely as the modified pilus probably would not recognize the receptor and therefore the organism would not be pathogenic. In addition, immunizing the mothers will provide only limited protection since the antibody titer is greatly reduced in non-colostral milk, even though, the milk supply to piglets will stop upon weaning. On the other hand vaccination of the piglets is of limited value due to the long time required for the immune system to respond to an antigen stimulation and produce specific antibodies that are needed at an early age (5- 21 days). Even if specific antibodies were produced, they will be of all isotypes, however, only the secretory type of IgA in its dimer form will pass through the epithelial cells to the mucosal surfaces of the intestine (Binjamini, and Leskowitz, 1991). Also the level of IgA would not be high, therefore, only a limited degree of protection would be conferred against intestinal diseases.

Post- weaning diarrhea may also be caused by ETEC other than *E. coli* K88, K99 or 987P.

Thus, antibodies against these antigens would be ineffective at controlling ETEC. Bianchi et al, (1996) for example showed that vaccination of piglets with *E. coli* K88 antigen did not provide protective immunity at the mucosal level in older piglets against PWD. This could be due to the diversity of diarrhea causing agents, in which K88 is only one of several possible causative agents. A new type of pilus adhesion named 2134P was identified by Nagy et al, (1992 a, b) and found to be a causative agent for weaning diarrhea.

Recent studies conducted on the efficacy of using fimbriae as vaccines have had great success with human culture cells and in mice. Langermann et al, (1997) reported that antisera from vaccinated animals against the Fim H of uropathogenic *E. coli* inhibited the binding of *E. coli* to human bladder cells in vitro. The colonization of the bladder was decreased by 99% when mice were immunized with Fim H and subsequently challenged with bacteria.

2.6.3 Adhesion blocking lectins and enzymes

The specific attachment of bacteria to mucosal surfaces is a pre-requisite to colonization of the host intestine by pathogenic bacteria. Carbohydrate or lipid conjugates found on the surface of the epithelial cells form the basis of this attachment. Several studies have been conducted using synthetic carbohydrates to block these attachments. Neeser et al, (1986) have prepared mannoside oligosaccharides from soybean, kidney beans and from ovalbumin and used them to inhibit the attachment of type 1 fimbriae to its receptors. Glycoprotein glycans were used by Mouricout et al, (1990) to inhibit the adhesion of *E. coli* K99 to the intestine. The glycans were derived from bovine plasma and proved to be effective in reducing the adhesion of *E. coli* to calf intestine, however, to be used effectively, large amounts (1.5-3 g) were needed for each calf. Thus, such an approach is

not practical. Further, it is not known whether pig plasma contains similar components that can be used for swine therapy and protection. Meng et al, (1997) using hemagglutination, demonstrated that some non-pathogenic bacteria such as *Bifidobacteria psuedolongum* have a 9-10 fold greater affinity for the binding of porcine intestinal epithelial cells than K88. Therefore, such bacteria can be fed to piglets, colonizing the entire intestine thus, inhibiting the binding of *E. coli* K88 or any other pathogen. Maynott et al, (1996) used a novel procedure to prevent diarrheal diseases caused by K88 *E. coli* in piglets. Their procedure is based on using proteolytic enzymes to digest K88 receptors rendering *E. coli* K88 unable to attach to small intestine. Bromelain, a proteolytic enzyme extracted from the pineapple stem, was fed orally to piglets. Its effect was dose dependant with the binding of *E. coli* activity being decreased with increasing concentration of enzyme. Mynott et al, (1991) used microencapsulated protease preparations to minimize the loss of the enzyme before reaching its site of action. However, such procedures are expensive and even difficult to apply and control especially when used on large scale pig farms.

2.7 Therapeutic antibodies

As discussed earlier, several approaches have been used to prevent or minimize the incidence of diarrhea in piglets, but all suffer major drawbacks or are simply impractical. An attractive alternative approach is the use of therapeutic antibodies to confer passive immunity.

One source of readily available immunoglobulins is from the blood of pigs obtained from slaughter plants. The plasma fraction is collected, spray dried and incorporated into the diet of early weaned piglets. Studies have indicated that the incidence of diarrhea is reduced significantly among piglets fed diets containing the plasma. This has been attributed to the presence of antibodies against

intestinal pathogens (Marquardt, 1997). In one of the first studies, Elliot, (1978) reported that the addition of immunoglobulins obtained from porcine blood to a milk replacer maintained 66% survival rate in piglets compared to none in the control group. Another study was conducted on horses to evaluate the effect of plasma from immunized horses against wild heat killed *E. coli*. A group of horses suffering form endotoxemia clinical signs were given serum from the immunized horses, while a control group was given pre-immune serum. The mortality rate was 13% in horses that were administered hyperimmune sera compared to 47 % for those given the pre-immune sera. In addition, the horses that received the hyperimmune serum experienced a shorter period to recovery and milder symptoms than those received the pre-immune serum (Spier et al, 1989). In a recent study, pigs were vaccinated against whole cell lysates of ETEC, serum then was collected and fed to piglets. The piglets were protected from diarrhea caused by the same ETEC compared to controlled piglets who consumed serum of non-immunized pigs (Isaacson, 1994). However, it seems that the pigs serum is widely used as a feed supplement where it was found to enhance the growth dramatically due its high protein content and to less extent as therapeutic agent against colibacillosis. One disadvantage of using the serum as therapeutic agent could be its high cost and the limited availability of such material. In addition, to be effective, it has to be administered in high amounts which also limits it use on a wide scale (Spier et al, 1989)

Monoclonal antibodies, have also been used for treatment of some diseases in domestic animals. Early trials to utilize specific monoclonal antibodies to treat calves infected with K99 diarrhea have been reported. Sherman et al, (1983) reported a significant decrease in both severity of the diarrhea and mortality (29%) for those given the monoclonal anti-K99 compared to 82% mortality for the control group. Smith and Lida, (1990) reported that piglets that were fed

monoclonal antibodies against *Actinobacillus pleuropneumoniae*, following a challenge with the same organism, were markedly protected against developing pleuropneumoniae, while those given saline died. Monoclonal antibodies were also used for short term prophylaxis and therapy against pseudorabies virus infections in swine (Marcholi et al, 1988). However, using monoclonal antibodies for therapy is prohibitively expensive and therefore, not practical for the large scale treatment of swine diseases such as diarrhea caused by ETEC.

2.8 Chicken egg yolk antibodies

The egg yolk is a rich source of antibodies (Grossman et al, 1990; Jensenius et al, 1981) . Like mammals, chicken also protects their progeny by passing the maternal antibodies form their serum to the egg yolk. Egg yolk antibodies are called IgY, and are different from mammalian IgG. Chicken IgY has a MW of 180 kDa with two subunits with the MW of the heavy chain being 67 to 70 kDa while that of light chain being 22-30 kDa. (Grossman et al, 1990). Chickens produce eggs non-invasively, and when vaccinated, the adjuvants does not cause severe reactions like mammals such as mice and rabbits. It is also possible to obtain antigen-specific chicken IgY with immunization of the desired antigen over a long period of time. Over 200-300 eggs per year can be obtained from one laying hen with approximately 150 mg pure antibody per egg (Marquardt, 1997). Further, the antibodies can either be purified by one of the several methods published in literature (Jensenius et al, 1981; Bade and Stegemann, 1984; Akita and Nakai, 1993; Clarke et al, 1993) or the whole egg yolk fraction can be spray dried or freeze-dried and used as a feed supplement (Marquardt, 1997).

2.8.1 Egg yolk IgY as therapeutic antibodies

Enhanced immunity by oral administration of antibodies with predefined specificity has been reported (Bartz et al, 1980; Shimizu et al, 1988; Yolken et al, 1988; Otake et al, 1991; Shimizu et al, 1993). Early attempts to use egg yolk IgY for protection goes back to the early 1980's. Bartz et al, (1980) reported the protection of infant mice against murine rotavirus by feeding egg yolk antibodies raised against this virus. The author reported that the treatment of challenged mice with egg yolk IgY, decreased the infection rate from 90% (non-treated mice) to only 15% for IgY treated mice. Similar results were obtained by Yolken et al, (1988). They produced egg yolk antibodies specific to rotavirus. The antibodies, when fed to mice were found to prevent both virus excretion and virus-induced gastroenteritis. In addition, egg yolk IgY from non-immunized chickens failed to prevent the development of rotavirus induced gastroenteritis in challenged mice.

Otake et al, (1991) demonstrated that rats can be protected against dental carries by incorporating egg yolk antibodies against *Streptococcus mutants* in the feed. The mean mandibular carries scores of rats challenged with *S. mutants* decreased by increasing the ratio of the specific IgY with the most significant reduction of carries being obtained when the egg yolk proteins constituted 20 % of the diet.

Complete passive protection of neonatal piglets against fatal enteric colibacillosis was reported by Yokoyama et al, (1992). Piglets treated with chicken antibodies raised against fimbrial adhesions of *E. coli* K88, K99, and 987P at a titer of 625 or 2500 had a complete (100%) survival rate after challenge with the corresponding strains of ETEC. Further, they reported that the piglets that received the antibodies at a titer of 2500 experience only mild diarrhea for a short duration of time. These results also demonstrated that antibodies are highly effective and that they exert their

effect in a dose dependant manner. Similar results were obtained by Ikemori et al, (1992). They reported that significant protection was achieved in all calves treated with IgY antibodies raised against *E. coli* K99 fimbriae. The survival rate was 100 % when high titers were used. In addition, no severe diarrhea and limited mortalities occurred among the challenged calves while a 100 % mortality occurred among the control group. Recently, Zuniga et al, (1997) conducted a study to confirm earlier reports on the efficacy of egg yolk antibodies for the control of intestinal colonization by *E. coli* F18 ab and ac in weaned piglets. They reported that full protection of piglets was achieved against these strains of *E. coli*. The effect was dose dependent with 5.5 g of egg powder per piglet providing a protective dose, while 3.5 g and less seemed to be insufficient for protection. It is note worthy that the presence of egg lysozyme and ovotransferrin may also function as antibacterial agents. However, such an effect would only be obvious when antibody-containing egg-yolk powder is used at high doses.

2.9 Anti-idiotypic antibodies

Antibodies under certain circumstances can be antigenic and induce an immune response to produce antibodies against themselves by xenogeneic (antibodies originating from a foreign species), allogeneic (having a genetic dissimilarity within the same species) and syngeneic (literally, genetically identical) immunizations (Benjamini, and Leskowitz, 1991; Pan et al 1995). Approximately 35 years ago Kunkel et al, (1963) and Oudin and Michael, (1963) referred to the unique antigenic determinants on the variable (V) domains of an antibody (Ab1) as idiotype (from the Greek roots of individual or personal). In addition, Oudin referred to the anti-antibody as an anti-idiotypic antibody (Kennedy et al, 1988). Normally there are several idiotopes in each V domain,

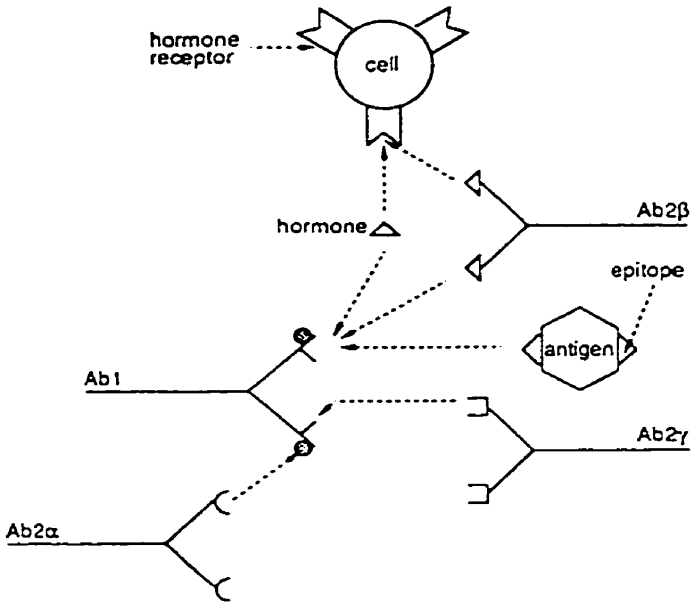
some are unique to the antibody termed private idiotopes, while others are shared with some other antibodies termed public idiotopes. (Nisonoff, 1991; Mayforth, 1993). Four types of antibodies (Ab2α, Ab2β , Ab2γ and Ab2ε) are normally produced when the antigen used in immunization is an antibody

Initially, anti-idiotypic antibodies (Ab2) were categorized by Jerne et al, (1982) into two classes, Ab2α and Ab2β. Later, Bona and Kohler, (1984) added third and fourth categories; Ab2γ and Ab2ε. Each of the four groups will be discussed briefly:

i) Ab2α are a set of antibodies that recognize idiotopes distinct from the antigen combining sites of Ab1. The binding of these antibodies to Ab1 does not alter the antigen binding capacity of Ab1. This set of antibodies is given the name anti-allotypic antibodies (Figure 2.1). They react with every antibody made by the individual in which Ab1 was obtained from (Bona and Kohler, 1984; Erlt and Bona, 1988; Kennedy et al, 1988; Pan et al, 1995).

ii) Ab2β is a type of antibody that carries an internal image of the nominal antigen. It recognizes idiotopes within the antigen combining sites of Ab1, thus, mimics the eliciting antigen. The anti-idiotypic antibodies share amino acid sequence with the protein antigens which accounts for some aspects of antigen mimicry. However, this does not explain the antigenic mimicry of some non-protein antigens. Such mimicry may involve less obvious similarities with less optimal degree of mimicry (Greenspan and Bona, 1993). Since these types of antibodies recognize their determinants in the hyper-variable region of Ab1, they are very specific to that region and do not react with other antibodies of the same class in an individual (Bona and Kohler, 1984; Erlt and Bona, 1988; Kennedy et al, 1988; Pan et al, 1995). Antibodies carrying an internal image of an antigen constitute

Figure 2.1: Idiotypes and anti-iditypic antibodies. Anti-idiotypic antibodies (Ab2) that recognize determinants on another antibody (Ab1) have been classified into three categories: Ab2α, recognizes idiotopes outside the binding site of Ab1; Ab2β, recognizes the binding site of Ab1 and is said to bear an internal image of the antigen recognized by Ab1; Ab2γ, recognizes the binding site but does not carry an internal image of the antigen (Adapted from Mayforth, 1993).


hormone
receptor
cell
hormone
Ab2β
epitope
antigen
Ab1
Ab2γ
Ab2α

only a small portion (15-20%) of the total anti-idiotypic antibodies produced in response to Ab1, however, they are considered to be an exceptionally useful experimental tool (Pan et al, 1995).

iii) Ab2γ, like Ab2β, recognizes idiotopes within the nominal antigen combining site. They can inhibit the binding of the antigen to Ab1. However , they do not bear an internal image of the nominal antigen. Therefore, these antibodies bind not only to Ab1 that induced their production but to other antibodies within the same class in an individual, thus, they were given the name anti-isotypic antibodies (Bona and Kohler, 1984; Kennedy et al, 1988; Mayforth, 1993; Pan et al, 1995).

iv) The last type of antibodies is designated Ab2ε. It is specific for antigenic determinants of Fab region in human IgM that exhibit anti-γ-globulin activity (Bona and Kohler, 1984).

In general, the specificity of the different types of anti-idiotypic antibodies are reflected in their ability to block the binding of Ab1 to its corresponding antigen. Ab2α does not block the binding of Ab1 to its antigen, Ab2β completely blocks this binding, while Ab2γ partially blocks this binding. It is not known whether Ab2ε interferes with Ab1-antigen binding (Chanh et al, 1992; Mayforth, 1993).

2.10 Conventional vaccines

The problems and hazards that usually accompany the use of conventional vaccines had led to the development of new vaccine alternatives. Traditionally, vaccines are produced by either inactivating the bacteria or virus or just attenuation of their virulence. However, attenuated bacteria or virus may revert back to a virulent state once injected into the host, thus, posing a risk of contracting the disease in immunocompromised as well as healthy people. In addition, dealing with highly virulent viruses and bacteria poses a major risk for laboratory personnel (Eichmann et al,

1987; Bhogal and Miller, 1991; Mayforth, 1993). Furthermore, some organisms can not be grown in vitro, thus, their mass production is not possible, while others evade the immune system by antigen switching or they produce carbohydrate antigens that fail to evoke the immune system (Eichmann et al, 1987). Therefore, due to the aforementioned hazards and obstacles in producing conventional vaccines, there is a need for a safe vaccine.

Recent advances in biotechnology have provided some other alternatives to conventional vaccines such as, synthetic peptides, recombinant proteins, recombinant microorganisms and anti-idiotypic vaccines (Eichmann et al, 1987; Bhogal and Miller, 1991). The first four types of vaccines will not be discussed since they are beyond the scope of this thesis. Only anti-idiotypic vaccines will be discussed.

2.11 Anti-idiotypic vaccines

Antibody molecules are very specific for a determinant on a given antigen. This specificity is determined by its variable (V) region which contains the antigen binding site. Antigenic determinants within the V region are called idiotopes and are unique for each single antibody (Dreesman and Kennedy, 1985; Rico and Hall, 1989). The specific region of an antibody that interacts with an antigen is named paratope. If this paratope and the idiotope have the same structural region on a given antibody molecule, then the conformational fit of the antigen and anti-idiotype antibody to the sites on the antibody should be a mirror image, thus the anti-idiotypic antibody is called a mimic of the antigen (Dreesman and Kennedy, 1985; Rico and Hall, 1989). Roitt et al, (1985) showed that an internal image does not mean that Ab2 carries the structure of the entire antigen site. Instead, it might carry only an image of particular epitope or few residues within the

epitope recognized by Ab1.

Immunization of a host with an anti-idiotypic antibody bearing the internal image of the antigen will evoke the immune system to produce antibodies against the antigenic determinants of this antibody (Ab3). The resulting Ab3 would be able to bind to the nominal antigen, thus, it could provide immunity to the host (Rico and Hall, 1989).

It appears that there is no limitations or specific choices for the antibody (Ab1) to which anti-idiotypic (Ab2) can be developed. Therefore, anti-idiotypic antibodies can be produced to mimic any substance that can meet the requirements for immunogenicity (Eichmann et al, 1987).

2.11.1 Clinical uses of anti-idiotypic antibodies as vaccines

Anti-idiotypic antibodies have been produced and utilized as protective agents against intracellular parasites, viruses, bacteria and their toxins. In addition, they have been used effectively in identifying cell surface receptors for several bacteria and viruses, and interestingly they were used to block pregnancy in mice (Chanh et al, 1992). Each of these application except the last, will be discussed with regard to the pertaining literature.

The first successful anti-idiotypic vaccines was reported against *Trypanosoma rhodesiense* (Eichmann et al, 1987). This strain is one of two parasites that cause sleeping sickness that affects over 50 million people in Africa. The researchers (Sacks et al, 1982), who conducted the study, reported that immunity acquired by challenge mice was one of three categories ; i) complete protection, ii) reduced parasitemia and iii) selection against parasites bearing the original variable antigen type. Another attempt to use an anti-idiotypic vaccine against parasites was reported by Grzych et al, (1985). Partial protection (50-76%) against an infection of *Schistosoma mansoni* in

rats was achieved by immunizing rats with anti-idiotypic antibodies bearing an internal image of a 38 kDa protein antigen of *S. mansoni.*

Viruses are peculiar organisms that require precautions and certain preparations for their growth. Thus, it is always difficult to obtain large quantities of a protective antigen that can be used to produce a vaccine against a particular virus. Recombinant DNA technology have solved parts of the problem, however, some of the viral protective antigens are glycoproteins that contain both protein and carbohydrate moieties where the carbohydrate part cannot be produced by such technology. Anti-idiotypic vaccines may overcome some of these obstacles.

Anti-idiotypic vaccines have been successfully used to immunize animals against certain viruses. Kennedy and coworkers, (1984,1986) produced rabbit anti-idiotypic antibodies against human antibodies that recognize hepatitis B surface antigen. The antibodies were tested for their vaccine potential in chimpanzees. The two vaccinated chimpanzees were protected against hepatitis B infection while the control chimpanzees were not protected and produced clinical and serological characteristics of hepatitis B disease. Anders et al, (1989) working with influenza virus, reported production of anti-viral antibodies by immunizing mice with anti-idiotypic antibodies. The second antibody was produced against five preparations of Ab1 which recognizes specific antigen sites on the hemagglutinin of the influenza virus. Two of these five preparations led to the production of neutralizing antiviral antibodies while the other three preparations failed to evoke the mice immune system to produce antiviral antibodies. Srikumaran, (1990) produced anti-idiotypic antibodies that induced the development of neutralizing antibodies (Ab3) to bovine herpes virus I. However, the titers of Ab3 in mice were not very high, nevertheless, they indicated that there was a true image of Ab2 in the idiotypic preparation used for the immunization. No clinical trials were preformed to

evaluate the efficacy of the Ab3 against a real challenge of mice with the virus. Griedr and Schultz, (1990) produced a monoclonal anti-idiotypic antibody that neutralized blue-tongue virus in vitro. It was found that the mAb inhibited the binding of the virus to its cellular receptor site.

Growing bacteria is generally not as difficult as growing viruses, however, some bacteria are highly virulent and dealing with them poses a risk to laboratory workers. In addition, some bacteria are very slow growers and need certain media requirements which makes growing them an obstacle in itself. Anti-idiotypic antibodies can be a good vaccine substitute for some bacterial antigens and generally will confer immunity in a target host.

Soon after the first report that detailed the successful use of anti-idiotypic antibodies as a vaccine against *Trypanosoma rhodesence*, several reports using anti-idiotypic vaccines against bacterial infectious agents appeared in the literature. Stein and Sodersrotm, (1984) were the first to report the ability of anti-idiotypic vaccines to protect against bacterial infection. They produced anti-idiotypic antibodies against the capsular polysaccharide of *E. coli* K13. Immunized mice were protected compared to non-immunized mice. Further, they have showed that anti-idiotypic antibodies administered to mothers within 24 h of delivery can pass through milk and prime the pups for passive protection. In the same year, McNamara et al, (1984) also produced a monoclonal anti-idiotypic antibody that conferred protection in mice against lethal *Streptococcus pneumonia* infection. The anti-idiotypic antibody however, was not protective against the infection when it was administered alone. It had to be conjugated to a protein carrier to activate the T-helper cells that conferred the protection. Polyclonal mouse anti-idiotypic antibodies that bear an internal image of *E. coli* fimbrial protein were produced by Paque et al, (1990). The anti-idiotypic antibodies failed to induce protective immunity against infectious *E. coli* in mice, however, they stimulated a

secondary immune response in the challenged mice. The failure was attributed to the fact that mice do not represent a satisfactory animal model for human urinary tract infections. Brossay et al, (1993) tried to exploit anti-idiotypic antibodies as a replacement for the very toxic lipopolysaccharide vaccine. Anti-idiotypic antibodies were produced in rabbits against a monoclonal that possessed bactericidal activity. The resulting anti-idiotypic antibodies were found to bear an internal image of the antigen. Further, they were able to elicit an anti-LPS immune response in mice when they were immunized with these antibodies. Tanaka et al, (1994) produced rabbit anti-idiotypic antibodies that mimicked a flagellar antigen in *Clostridium chauvoei*. This pathogen causes black leg which is a fatal disease in cattle, sheep and other ruminant animals. Mice that were immunized with the anti-idiotypic antibodies had a survival rate that was twice that of the control mice when challenged with the pathogen.

Not all bacterial infections are similar in terms of growth and disease causing mechanism in the host. Some bacteria attack certain cells such as macrophages in the host and inhibit these cells. This enables the bacteria to evade direct contact with the immune system, thus, a proper immunity will not be developed against such pathogen. Immunization against such bacteria will be of particular importance. Immunization with the whole organism mainly induce the humoral arm of the immune system, and in several cases produces host hypersensitivity reactions. Other trials with synthetic peptides have not always been efficient (Borssay et al, 1994). Anti-idiotypic antibodies might provide a solution to the problem as it could function as surrogate antigens to induce protective immunity.

Listeria monocytogenes is a facultative intracellular pathogen that causes listeriosis which is an infection in humans. Vaccination against infections by this pathogen needs a live bacteria to

induce proper immunity. However, live vaccine causes several problems which make the anti-idiotypic vaccine a good candidate. Early in 1985, Kaufmann and coworkers, produced anti-idiotypic antibodies that recognize a monoclonal antibody against *L. monocytogenes* and induced protective immunity against *L. monocytogenes* in mice. The efficacy of the vaccine was reported to be lower than that of the live bacterial vaccine. Beauclair and Khansari, (1990) developed anti-idiotypic antibodies against a protective determinant on the intracellular pathogen, *Brucella abortus* . Mice immunized with this antibody were protected when challenged with an intraperitoneal dose of a virulent strain of *B. abortus*. Further, more than a 90% reduction of bacteria was observed in the spleen of challenged mice compared to the unvaccinated control group.

2.11.2 Vaccines against naturally occurring toxins

Naturally occurring toxins are highly lethal, some are non-proteineous and have small molecular weight. These characteristics have made the development of protective vaccines against them difficult and risky at the same time (Chanh et al, 1992). Therefore, antibody-based vaccines may be the only safe and effective method for designing new vaccines against such highly toxic substances. Anti-idiotypic antibodies have proved to be good substitute for traditional vaccines against many highly infectious viruses and bacteria. However, with toxins anti-idiotypic vaccines may represent the best choice.

Diphtheria toxin is a protein with a MW of 58 kDa and has three subunits. It is produced by *Bordetella pertussis,* is highly toxic and poses certain risks in children at an early age. In an attempt to explore the possibility of producing an antibody-based vaccine, Rolf et al, (1989) produced an anti-idiotypic antisera that bears an internal image of one of the subunits of the toxin. The anti-

idiotypic serum protected vero cells from the action of the toxin, however, the antibody did not prevent the toxin from binding to the cells. Indeed, the anti-sera prevented the internalization and degradation of the toxin. The results indicated that the idiotypic antisera bear an internal image to the neutralization site of the toxin but not to the binding site. No further *in vivo* studies with an animal model have been reported.

Percival et al, (1990) produced anti-idiotypic antibodies that bore an internal image of D toxin that is produced by *Clostridium perfringens*. In contrast to the previous study by Rolf et al, (1989) the monoclonal anti-idiotypic antibody appeared to recognize the receptor as it prevented the toxin from binding to guinea pig peritoneal macrophage cells. However, treatment of the cells with the anti-idiotypic monoclonal antibody did not provide complete protection due to the fact that the fraction of anti-idiotypic antibodies that bears internal image is very small and its affinity to the receptor might not be high enough to block all the receptors. Animals that were challenged with toxin and were previously immunized with the anti-idiotypic antibody were protected against the toxin. In addition, they were protected when challenged with a dose of vegetative cells.

The trichothecene mycotoxin, T-2, is a highly potent none proteineous toxin with a low MW (≈ 466 Da), thus, it cannot be used as an immunogen in a conventional vaccination protocol. Therefore, a anti-idiotypic vaccine seems to offer an alternative solution. Chanh et al, (1990) produced and characterized a murine monoclonal anti-idiotypic antibody that seemed to be protective against the effect of T-2 toxin when tested both *in vivo* and *in vitro*.

2.12 Clinical uses of anti-idiotypic antibodies as therapeutic agents

All the above mentioned studies were directed toward immunizing a host with anti-idiotypic

antibodies bearing an internal image of certain pathogen or toxin. However, only two studies in the literature have addressed the use of anti-idiotypic antibody as a therapeutic agent rather than as a vaccine. Jackson et al, (1990) demonstrated that administration of rat anti-idiotypic antibodies provided partial protection against dental carries in rats. The antibodies which bear an internal image of an antigenic determinant on *Streptococcus mutants* were packaged in liposomes and administered parenterally to the challenged rats. In addition to a reduction in carries, colonization of the oral cavity by *S. mutants* was reduced. Reck et al, (1988) and Bamberger et al, (1986) produced a monoclonal anti-idiotypic antibody against *Staphylococcus* enterotoxin B. Interestingly, the anti-idiotypic antibody was administered through gastric tubing directly into the stomach and conferred complete protection against emetic response and diarrhea in monkeys challenged with the toxin even at a dose of 100 pico moles.

Anti-idiotypic antibodies have also been reported to act as antimicrobial agents. Polonelli et al, (1993) reported that mouse anti-idiotypic antibody mimicked the action of yeast killer toxin produced by *Pichia anomala* as it was effective in killing strains of *Candida albicans*.

2.13 Anti-idiotypic antibodies as probes for receptors

The first and perhaps the most critical step in an infection is the recognition of the host cell receptors by the pathogen. The presence or absence of specific cell receptors is the basis of host specificity for different infectious agents (Thaker et al, 1994). Molecular characterization of these receptors has been an attractive challenge for researchers for several years. The scarce amounts of these receptors and the difficulty in their extraction has made their identification and characterization extremely difficult. Several polyclonal and monoclonal antibodies against partially or highly purified

receptors have been reported, but as mentioned before the difficulty in preparing amounts sufficient for immunizing a host and the peculiarity of their nature has limited the use of this method (Glasel and Myers, 1985; Couraud and Strosberg, 1991; Nisonoff, 1991). Anti-idiotypic antibodies have been exploited effectively as a specific markers to identify, isolate and block a variety of specific cell surface receptors for bacteria, viruses and hormones (Gaulten et al, 1985).

The first use of anti-idiotypic antibodies as markers of cell surface receptors was conducted by Sege and Peterson, (1978). The authors produced anti-idiotypic antibodies against antibodies that recognized retinol binding protein (RBP). The antibodies specifically bound rat intestinal epithelial cells that had a cell surface receptor for RBP and were able to inhibit uptake of retinol by the cells in a concentration dependant manner. In addition, the authors also reported that an anti-idiotypic antibody against antibodies that recognized insulin was able to block the binding of ^{125}I-labelled insulin to rat epidermal fat cells. These results indicated that anti-idiotypic antibodies have an internal image of the RBP and insulin ligands as they recognize and bind their receptors.

Noseworthy et al, (1983) and Kauffman et al, (1983) reported that monoclonal and polyclonal anti-idiotypic antibodies against the idiotype of an anti-reovirus types 3 hemagglutinin were highly effective in binding reovirus cell surface receptors as they were able to block the binding of the virus to such cells. Further, the antibodies were used effectively to identify a cell surface receptor for the reovirus. Cellular receptor for bovine herpes virus I was also identified by using a bovine anti-idiotypic antibody produced against a monoclonal antibody that recognized a virus glycoprotein IV (gIV). The anti-idiotypic antibodies were radio-labeled and used to identify the putative virus receptors using immunoblotting of T-cell membrane extracts that were separated by SDS-PAGE electrophoresis. The anti-idiotypic antibodies recognized a 60 kDa protein which is

believed to be the receptor for the virus.

Rabbit anti-idiotypic antibodies that recognize a Fab fragment of a monoclonal antibody against morphine was reported by Glasel and Myers (1985). The anti-idiotypic antibodies inhibited the binding of morphine to rat brain opiate receptors in a concentration dependant manner. This implied that the anti-idiotypic antibody mimicked morphine and competed with it in the binding of its receptors. Several other reports using anti-idiotypic antibodies for receptor identification were published in the early 1990's. Chua et al, (1989) identified the atrial nattriuretic factor using SDS-PAGE electrophoresis and rabbit anti-idiotypic antibodies for immunoblotting analysis. Czop et al, (1990) reported the production of rabbit anti-idiotypic antibodies that recognized the human monocyte receptor for yeast B-glucans. Glutamate receptors were identified and studied in vertebrates by Duec et al, (1991) using rabbit anti-idiotypic antibodies. Recently, Pchelintseva et al, (1995) used anti-idiotypic antibodies from rabbits and chicken for the identification of protein receptor components for pre-aldehyde dehydrogenase using SDS-PAGE electrophoresis and immunoblotting. Using both types of antibodies, several proteins (29, 34, 42, 60, and 66 kDa) that could be putative receptors were identified.

The above mentioned studies and several others in literature suggest that anti-idiotypic antibodies provides a highly effective method for receptor identification and characterization and may be the method of choice due to the relative ease of producing and purifying such agents.

2.14 Advantages and draw backs of anti-idiotypic antibodies as therapeutic agents

2.14.1 Advantages of anti-idiotypic antibodies: There are several merits of using anti-idiotypic vaccines over traditional vaccines include the following : i) they can be used to prevent diseases

caused by pathogens that cannot be grown *in vitro* such as viruses and some dangerous and extremely difficult to grow bacteria such as *Mycobacterium liperae* and *Legionella* (Dreesman and Kennedy, 1985; Ertl and Bona, 1988) or against microbes exhibiting antigenic variation such as influenza virus and HIV or *Trypanosoma* parasite (Ertl and Bona, 1988; Rico and Hall, 1989; Mayforth, 1993) ; ii) anti-idiotypic antibodies eliminate the complications of using whole killed or attenuated organisms that might cause immune problems due to their complex surface antigens, such as lipid A which is highly toxic. In addition, some pathogens such as *Streptococcus pyogenes* have several antigens on their surface, in which one of these determinants resembles a protein in myocardial muscle, thus, such vaccine will evoke the immune system to produce antibodies that recognize this antigen in both bacteria and heart muscle creating fatal problems (Dreesman and Kennedy, 1985; Kennedy et al, 1988; Mayforth, 1993); iii) the ability of anti-idiotypic antibodies to trigger both B and T- cells is a major advantage. The ability of these vaccines to induce T-cell response may help in inducing immunity in neonates who do not usually respond to conventional vaccines, and carbohydrate vaccines such as those present in *Haemophilus influenza, Neisseria meningitis* and group B *Streptococcus* (Kennedy et al, 1988; Rico and Hall, 1989; Bhogal and Miller, 1991; Nisonoff, 1991 Mayforth, 1993) ; iv) anti-idiotypic antibodies have proven to be beneficial when the epitope is carbohydrate, lipid or DNA which are difficult to mass produce , or poor immunogens and in case of carbohydrates and lipids cannot be produced by recombinant DNA technology (Kennedy et al, 1988; Mayforth, 1993) ; v) attenuated pathogens might revert back to their virulent stage, thus cause the disease in vaccinated individuals (Bhogal and Miller, 1991); vi) mapping of antigenic sites is considered one of the big advantages of idiotypic antibodies (Rico and Hall, 1989); and finally, vii) anti-idiotypic antibodies have been shown to be effective as

immunizing agents against certain cancers (Kennedy et al, 1988).

2.14.2 Drawbacks of anti-idiotypic antibodies: Although anti-idiotypic vaccines have several advantages, they have their own limitations which restricts their use in some cases. Their major limitations are: i) the use of anti-idotypic vaccines produced in one species for use in other species can cause serum sickness due to the phylogentic differences between the host and the source of antibody. This is mainly true when multiple injections are needed (Dreesman and Kennedy, 1985; Rico and Hall, 1989; Chanh et al, 1992; Mayforth, 1993). However, the problem of development of serum sickness might be minimized by introducing human heavy chain constant region into the rodent or avian antibodies by genetic engineering; i.e, humanizing the antibodies. This will probably add more expenses to the development of such vaccines (Nisonoff, 1991). This problem, however, does not arise if the antibodies are fed or administered orally to animals or humans ; ii) some anti-idiotypic vaccines may elicit a response equivalent to the nominal antigens but some others have been found to suppress the immune system (Rico and Hall, 1989; Bhogal and Miller, 1991); iii) one of the concerns about anti-idiotypic immunity is that it might be of shorter duration than the immunity induced by the nominal antigen (Mayforth, 1993) ; iv) immunization with anti-idiotypic vaccines may not confer complete protection, thus, a booster injection with the nominal antigen might be required (Rico and Hall, 1989); and v) a simple antigen or a peptide can elicit the production of a diverse collection of antibodies (Ab1) that can be a template for the production of Ab2. Nevertheless, it was found that only some of these templates might be structurally capable of eliciting an antibody (Ab2) that bears an internal image of the antigen (Mariazza and Poljak, 1993).

2.15 Molecular stability of chicken IgY

Chickens, similar to other mammals passes immunoglobulins to their embryos for protection. On average, an egg can have up to 150 mg of immunoglobulines that are called IgY (Shimizu et al, 1992). Chicken IgY possess many characteristics that are different from the IgG of most mammals. It does not bind to complement nor to the rheumatoid factor. In addition, anti-mammalian IgG does not cross react with chicken IgY. The above mentioned characteristics of the IgY makes it useful particularly when used as a biological and/or a clinical reagent (Shimizu et al, 1992). Also it is not necessary to purify egg-yolk IgY when used as a therapeutic agent against a certain disease. Oral administration of specific antibodies against bacteria or virus through an infant formula might provide an effective method of preventing intestinal infections (Shimizu et al, 1993). However, oral administration means that the IgY has to pass through the stomach to the intestine where it acts, without being digested or inactivated by the stomach acidity and the peptic enzymes. Several studies have been conducted in vitro to explore the stability of IgY against acid and peptic digestion. Shimizu et al, (1988) studied the stability of anti-*E. coli* chicken IgY under different conditions. The chicken antibodies were evaluated at pasteurization temperatures, low pH, and in the presence of proteolytic enzymes. It was found that IgY was relatively heat stable at pasteurization temperature, however, it was more susceptible to pepsin digestion than bovine IgG with a significant loss of activity occurring when digested by pepsin at pH 4.5. In addition, IgY was found to be less susceptible to trypsin and chymotrypsin digestion. Shimizu et al, (1992, 1993) studied the molecular stability of chicken IgY and compared it to that of rabbit IgG in one study and to cow, goat and pig antibodies in another study. Chicken IgY was found to be more susceptible to acidic treatment than rabbit IgG. Destruction of the secondary structure of IgY was found to proceed more

quickly than that of IgG from mammals. In addition, heat denaturation of chicken IgY was greater than that of the rabbit IgG but was similar to that of cow, pig and goat IgG. A similar study was conducted by Otani et al, (1991). They reported that IgY was fairly stable after heating up to 60°C for 10 min. In addition, it was found to be stable in neutral, alkaline and acidic conditions. It was also found to be resistant to proteolysis especially in the presence of trypsin and chymotrypsin but was slightly more susceptible to pepsin. However, chicken IgY was reported to be more susceptible to proteolysis than rabbit IgG. The difference in susceptibility might be due to localization of disulphide bonds or presence of different amounts of carbohydrate in the proteins. Chicken IgY has less covalently bound carbohydrate than that of IgG. Carbohydrates have been shown to stabilize proteins against denaturation. Finally, the effect of high sugar concentrations and high pressure on IgY stability was studied. Shimizu et al, (1994) reported a 10-20% loss in chicken IgY activity when treated at high pressures at various temperatures. A high sugar concentration was found to suppress inactivation of the IgY by heat, acidic or pressure treatment. In the presence of 50% sucrose, IgY retained complete activity when heated at 80° C for 15 min. In addition, at > 30% sucrose or 20 % dextran, IgY was completely stable at pH 3 . The addition of different sugars increased the stability of IgY during storage irrespective of the concentrations that were used.

The above studies demonstrated that IgY is a fairly stable antibody under non-harsh conditions. This stability was enhanced by the addition of different amount of certain sugars. However, it might not be possible to add high amounts of sugar to the antibody preparation if it is to be used in an infant formula or for other therapeutic purposes. Encapsulation, of the IgY by liposomes seems to be a promising method that can shield the antibodies from the acidity and peptic hydrolysis when administered orally. Shimizu et al, (1993) encapsulated IgY in liposomes by using

a dehydration-rehydration method. The encapsulation reduced the loss of activity of IgY antibodies under both acidic and peptic hydrolysis. Unfortunately, in the process of encapsulation, certain chemicals such as formaldehyde have been used which might limit the use of encapsulated antibodies in food fortification .

3. Manuscript I

Use of Chicken Anti-idiotypic Antibodies for the Treatment of Diarrhea Caused by *Escherichia coli* K88 Infection

3.1 Abstract

Polyclonal anti-idiotypic antibodies (Ab2) were produced in laying hens against three monoclonal (mAb1) and three polyclonal (pAb1) antibodies that recognized *Escherichia coli* K88 fimbrial antigen. The Ab2 were tested for the presence of antibodies that bear an internal image of K88 fimbriae using indirect competitive ELISA. The Ab2 produced against anti-K88 pAb1 inhibited binding of K88 fimbriae to pAb1 by 28-35 % while Ab2 produced against anti-K88 mAb1 inhibited the binding by only 6-15 %. Pure Ab2 of all preparations were injected into mice to produce Ab3 and the serum was tested by indirect ELISA to determine if it recognized the K88 antigen. The results demonstrated that Ab3 was able to recognize K88 fimbriae with the reaction being stronger for Ab2 derived from anti-K88 rabbit pAb1 than that of Ab2 derived from the corresponding mAb1. However, similar non-specific titers were obtained for both types of preparations. The immunized mice were also challenged with an IP injection of 5 $X10^8$ CFU of *E. coli* K88. The Ab3 present in the serum produced complete protection against death (100% survival) in two experiments. In contrast, the survival rate in the non-immunized mice from the two experiments were 25 and 18%. A second study was undertaken to evaluate the passive efficacy of two Ab2 preparations (Ab2 produced against rabbit anti-K88 IgG2a and its $F(ab')_2$) when administered orally to neonatal piglets challenged with *E. coli* K88. Partial protection (50 % survival rate) was observed in the Ab2 treated piglets, while only 13.5 % of control piglets (no-Ab2) survived. Our results indicates that Ab2 not only are excellent surrogate antigens for immunization, but can also provide considerable protection when used for passive immunization.

3.2 Introduction

Diarrhea caused by enterotoxigenic *Escherichia coli* K88 (ETEC) is one of the most prevalent diseases in neonatal piglets worldwide (Yokoyama et al, 1992). The ability of ETEC to colonize the small intestine is a virulence factor as it is a prerequisite for the production of diarrhea in both humans and animals (Erickson et al, 1992; Nystrom, 1995). This colonization is facilitated by the binding of bacterial fimbriae to specific receptors on the villus epithelium (Klemm, 1985; Hacker, 1992; Smith, 1992).

Antibiotics have been widely used for the last few decades and are considered the therapy of choice for the control of the ETEC diseases. However, their use on a routine basis has contributed widely to the appearance of multiple drug resistance in bacteria such as *Salmonella* and *E. coli* (Hayes, 1986). Enhanced immunity by oral administration of antibodies with predefined specificity derived from serum (Elliot, 1978; Spiere et al, 1989), colostrum (Shimizu et al, 1988) or monoclonal antibodies (Smith and Lida, 1990; Sherman et al, 1983) have been used to combat certain intestinal diseases. However, it is prohibitively expensive to obtain large amounts of these antibodies or simply it is impractical (Yokoyama et al, 1992). The egg yolk has been shown to be a good source of antibodies. It is possible to obtain antigen-specific chicken IgY by immunizing laying hens with the desired antigen (Bartz et al, 1980; Yolken et al, 1988; Otake et al, 1991; Yokoyama et al, 1992). This convenient source of antibodies could replace the need for serum, colostrum or monoclonal antibodies. Chicken IgY has been successfully used against murine rotavirus infection in mice (Bartz et al, 1988; Yolken et al 1988), dental carries caused by *Streptococcus mutants* in rats (Otake et al, 1991), neonatal diarrhea caused by *E. coli* K88, K99 or 987P in piglets (Yokoyama et al, 1992; Ikimori et al, 1992; Marquardt et al, 1999) and *E. coli* F18

infections in weaned piglets (Zunga et al, 1997). Therapeutic antibodies that are currently available are mainly produced by immunizing the host with attenuated or inactivated bacteria or virus which often require elaborate and expensive purification. In addition, these pathogens possess certain risks to the working personnel and the complex nature of the bacterial outer membrane proteins and lipopolysaccharides might cause severe reactions at the site of injection in the animal.

Numerous studies have provided evidence that anti-idiotypic antibodies (Ab2) that bear an internal image to a particular antigen can be produced by immunizing a host with the antibody (Ab1) that recognize the antigen (Jerne, 1974; Rico and Hall, 1989). The portion of Ab2 that bear an internal image to the antigen can induce an antigen specific immune response in individuals that have never encountered the antigen (Ertl and Bona, 1988; Rico and Hall, 1989). Anti-idiotypic antibodies have been used to induce protective immunity to schistosomiasis, trypanosomiasis, *Nesseria gonorrhea*, *E. coli* K13, herpes virus, influenza virus, *Streptococcus mutants*, *Listeria monocytogenes* and *Brucella abortus* (Brossay et al, 1993; Sacks et al, 1982, Crzych et al, 1985; Kennedy et al, 1986; Anders et al, 1989; McNamara, et al, 1984; Kaufmann et al, 1985; Beauclair and Khansari, 1990). Despite the large number of reports on use of anti-idiotypic antibodies as a vaccine, there are only two reports that have addressed the use of anti-idiotypic antibodies as therapeutic agents or for passive immunization. Reck et al, (1988) and Jackson et al, (1990) demonstrated that anti-idiotypic antibodies bearing an internal image to *Staphylococcus* enterotxin B and to an antigen on *Streptococcus mutants* provided partial protection against emetic response and diarrhea in monkeys and dental carries in rats, respectively. This study was designed to determine if egg-yolk anti-idiotypic antibodies that bear an internal image to the K88 fimbriae can interfere with the colonization of *E. coli* K88 in the pig intestine by blocking its receptors and

therefore prevent infection by this organism. The ability of anti-K88 Ab3 to protect mice against a challenge with *E. coli* K88 was also investigated. In this paper, monoclonal antibodies (mAb1) was produced in mice while polyclonal antibodies (pAb1) was produced in rabbits, pAb1 or mAb1 are the idiotype antibodies produced against K88 fimbriae, Ab2 are anti-idiotypic antibodies produced in chickens against pAb1 or mAb1 and Ab3 are polyclonal anti- anti-idiotypic antibodies produced in mice against Ab2.

3.3 Materials and Methods

3.3.1 Animals

Female Balb/c mice, 6-8 wk. of age were obtained from the Central Breeding Facility, University of Manitoba, Winnipeg. Twenty-week old Leghorn chickens were from the University of Manitoba poultry barn Winnipeg, and White New Zealand rabbits were from the Blue Farm Rabbitry, Winnipeg, Manitoba. Three days old Cotswold piglets were obtained from a local farm, Winnipeg, Manitoba. All animals were cared for according to the guide lines established by the Canadian Council on Animal Care (CCAC, 1980).

3.3.2 Bacteria and culture conditions

E. coli K88 that was cultured on trpytic soy agar slants was obtained from the Pennsylvania State University *E. coli* Reference Center (University Park, PA). *E. coli* K88 was grown in 8 L tryptic soy broth (TSB) (Difco laboratories, Detroit, MI) for 36 h at 37°C. Cells were then harvested by centrifugation, washed and used immediately for extraction of fimbriae. Alternatively, harvested bacteria were suspended in physiological saline and their concentrations were estimated by OD at

640 nm then used to challenge immunized mice. For the piglet experiment, a loopfull of K88 stock was inoculated into 250 ml TSB and incubated for 18 h at 37°C. This broth (200 μl) was spread onto blood agar plates (BAP) (Atlas Laboratories, Winnipeg, Canada) and incubated for 20-24 h at 37°C. Bacteria from BAP (3/pig) were removed by scraping, suspended in saline and used for the challenge studies. Absorbance of the suspension was determined at 640 nm as a measure of CFU numbers.

3.3.3 Extraction and purification of K88 fimbriae

Purification of K88 antigen was done according to the method of Erickson et al, (1992) with modification. Briefly, bacteria were cultured as described above in TSB for 36 h at 37°C. They were then harvested by centrifugation at 3000 X g for 15 min, washed once with 0.01 M PBS (pH 7.2), resuspended in the same buffer and heated in a water bath for 30 min at 60°C to release the fimbriae. While still hot, the bacteria were blended for 10 min at high speed in IKA-Ultra-Turrax T-25 homogenizer (IKA Laborotechnik, Staufen, Germany). Bacterial cells were removed by centrifugation (14,000 X g , 15 min), the supernatant was filtered through a 0.45 μm syringe filter (Nalgene, Rochester, NY) and fimbriae were precipitated by gradually adding 2.5 % citric acid until pH reached 4.0. The fimbriae were incubated for 2 h at 4°C, centrifuged at 14,000 xg for 30 min and the precipitation procedure was repeated three more times. Purity of fimbrial extract was determined by SDS-PAGE electrophoresis followed by silver staining.

3.3.4 Anti-K88 monoclonal antibody (mAb1) production and purification

Antibody producing cell lines were obtained from fusion of spleen cells of mice immunized

with pure K88 fimbriae and myeloma cells (P3x63-Ag8.653) using polyethylene glycol MW 4000 (Merck, Darmstadt, Germany) as described previously by Jaradat and Zawistowski, (1996). Antibody producing hybridomas were screened by ELISA using microtiter plates coated with K88 antigen. Several anti-K88 mAbs were produced and isotyped using a mouse isotyping kit (BioRad, Hercules CA). Antibodies were produced by inducing ascites in mice and purified using either protein-A Sepharaose for IgG2b following a procedure described by Harlow and Lane, (1988) or Fast Protein Liquid Chromatography (FPLC) gel filtration for IgM.

Samples (0.5 ml) of the IgM preparation were applied onto the FPLC Superose 12HR 10/30 gel filtration column (30 X 1.5 cm) that had been equilibrated with 0.05 M phosphate buffer (pH 7.2) at room temperature. Antibodies were eluted at a flow rate of 0.4 ml/min using the same buffer and elution was monitored at A_{280}. Fractions were collected, tested for activity by ELISA and for purity by SDS-PAGE electrophoresis, and used for subsequent experiments.

3.3.5 Production and purification of anti-K88 polyclonal antibodies (pAb1) and their F(ab')$_2$ fragments

White New Zealand rabbits were injected 5 times with pure K88 fimbriae emulsified in CFA for first injection and IFA for subsequent injections. Serum was collected and fractionated to IgG1, IgG2a and IgG2b using protein A-Sepharose column chromatography according to the method of EY et al, (1978) with modification. Briefly, serum (4 ml) was applied to the column (10 X 1.5 cm) at pH 8.0. Only IgG antibodies were bound to protein-A, while other immunoglobulins and serum proteins passed through the column. The column was washed with 30 ml of 0.1 M phosphate buffer (pH 8.0). IgG1 was eluted using 0.1 M citrate buffer (pH 6.0) followed by IgG2a at pH 4.5 and

IgG2b at pH 3.5. Fractions were collected and tested for activity by ELISA and for purity by SDS-PAGE electrophoresis.

$F(ab')_2$ fragments were prepared from the purified rabbit IgG2a. About 20 mg of pure IgG2a were suspended in 0.1M citrate buffer (pH 4.2) and pepsin was added at a ratio of 1:33 enzyme/substrate ratio (Coligan et al, 1992). The mixture was incubated for 8 h at 37°C and the reaction was stopped by adjusting the pH to 8.0 with 2 M Tris-base. The $F(ab')_2$ fraction was then purified using FPLC Superose 12 HR 10/30 gel filtration column and each fraction was tested for activity against K88 fimbriae by ELISA.

3.3.6 Production of anti-idiotypic antibodies (Ab2) in chicken

Twenty week-old white Leghorn laying hens were immunized with three mAb1, two rabbit pAb1 and $F(ab')_2$ of rabbit IgG2a that were purified. Initially, the birds were injected IM with 250 μg of the immunogen (1 ml) emulsified in CFA. In the second and subsequent injections each of 250 μg of immunogen were emulsified in IFA and administered biweekly. After the fifth injection, serum was tested for titer and the eggs were collected daily. Antibodies from serum were purified by FPLC using Superose 12HR 10/30 gel filtration column as for IgM. Alternatively, egg-yolk was separated from egg-white, and freeze-dried using a VarTis freeze dryer model Genesis 25 LE (Gardiner, NY). The titer of Ab2 in freeze-dried egg powder was measured by an indirect ELISA and found to be $> 10^6$ for all the Ab2 preparations.

3.3.7 Mice immunization for the challenge study

FPLC purified preparations of different anti-idiotypic antibodies (Ab2) were used for immunization. Eight groups of 6 Balb/c mice were immunized subcutaneously with 200 μl of a suspension containing 40 μg pure antibody or pure K88 fimbrial antigen emulsified in CFA. Equal amounts of antigen emulsified in IFA were given IP at two wk intervals in the second and subsequent injections.

3.3.8 Mice challenge with E. coli K88

All vaccinated and non-vaccinated mice were challenged with an IP injection of 5 X 10^8 CFU of *E. coli* K88 in 200 μl of PBS 1 wk after the last antigen injection. The mice were then observed for signs of infection every 2 h. Those mice that appeared to suffer from a severe infection were killed by CO_2.

3.3.9 Pig challenge with E. coli K88

The procedure was similar to that described by Marquardt et al, (1999). Thirty seven 3-day-old Cotswold piglets that were obtained from a local farm and were transferred to the University of Manitoba's Animal Research Unit. The piglets were fed milk replacer (skim milk, 109 g; whey powder, 77 g: methyl cellulose, 4 g; calcium carbonate and phosphate, 1 g each; water soluble vitamin mix, 4 g; and oil mix 35 ml, per one liter). Piglets were randomly divided into four groups; group I with 13 piglets was left without any treatment as the negative control. The other 3 groups containing 8 piglets each, were challenged with 10^{10} CFU of *E. coli* K88 in 5 ml of saline at zero time. Five hours before *E. coli* challenge, two groups were given Ab2 produced against either IgG2a

or its $F(ab')_2$ fragments, while the third group was left without treatment as a positive control. The experiment was conducted for 72 h with antibodies (2 g of egg yolk suspended in water and containing an antibody titer of $>10^6$) being administered to each piglet in the treated groups every 6-8 h (total 9 times). Both *E. coli* and anti-idiotypic antibodies (Ab2) were delivered directly to the stomach using a syringe and a polyethylene tube. The clinical responses of the piglets were monitored throughout the experiment for the occurrence of diarrhea, weight loss and mortality. Diarrhea scoring was as outlined in the footnote of Table 3.1 .

3.3.10 Immunochemical Methods

3.3.10.1 ELISA for testing avidity of monoclonal and polycolnal antibodies to K88 fimbriae: The avidity of the purified monoclonal and polyclonal antibodies to K88 fimbriae was evaluated by an indirect non-competitive ELISA. Microtiter plates were coated with 100 μl of 10 μg/ml of K88 fimbriae in carbonate buffer, pH 9.6 and incubated over night at 4°C. The plates were washed six times with PBST and then blocked with 200 μl of 5 % skim milk in PBST and incubated for 2 h at 37°C. After a washing step, 200 μl containing 100 μg of each purified antibody were added to the first well of the plate, serially diluted with PBS and incubated for 1 h at 37°C. The plates were washed and 100 μl of alkaline phosphatase conjugated goat anti-mouse or goat anti-rabbit antibody diluted 1:5000 in PBST was added to each well, and incubated for 1 h at 37°C. Alkaline phosphatase substrate solution (100 μl of 1 mg /ml of p-nitrophenylphosphate in diethanolamine buffer, pH 9.8) was added to each well and the plates were incubated at 37°C for 30 min. The OD was measured at 405 nm using a microplate reader model 450 (BiorRad).

3.3.10.2 Competitive ELISA for detection and quatitation of anti-idiotypic antibodies (Ab2) in chicken serum: The detection and quantitation of anti-idiotypic antibodies that bear an internal image to fimbriae was performed by an indirect competitive ELISA. Plates were coated with 1 μg/well of Ab1(IgG, IgG2a or its F(ab')$_2$) fragments from rabbits, or IgG2b, IgM1 or IgM2 from mice), diluted in 0.05 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. After blocking the plates with 5% skim milk, a mixture of serially diluted K88 fimbriae and a fixed amount (0.4 μg/well) of purified chicken serum were added to each well and incubated for 1.5 h at 37°C. Alkaline phosphatase-conjugated rabbit anti-chicken and ρ-nitrophenyl phosphate (ρNPP) were added as secondary antibody and substrate, respectively. The color was developed by incubating the plates for 30 min at 37°C and OD was read at 405 nm using a microplate reader model 450 (BioRad). The percent inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = \frac{\text{OD w/o inhibitor-OD with inhibitor}}{\text{OD w/o inhibitor}} \text{ X } 100$$

3.3.10.3 Analysis of serum antibodies (Ab3) by ELISA: The production of Ab3 (anti-anti-idiotypic antibodies) in mice was detected by indirect non-competitive ELISA. Microtiter plates were coated with 1 μg/well of purified K88 antigen or Ab2 diluted in 0.05 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing and blocking, 2-fold serially diluted serum from each group was added to each well and incubated for 1 h at 37°C. Color was developed by adding goat anti-mouse alkaline phosphatase conjugate as the second antibody and ρNPP as the substrate.

3.3.10.4 Sandwich ELISA: The presence of *E. coli* K88 in the fecal swabs of piglets before and after

the challenge was evaluated by a sandwich ELISA. Microtiter plates were coated with 100 μl of affinity purified anti-K88 rabbit IgG2a (10 μg/well diluted in carbonate buffer, pH 9.6) and incubated overnight at 4 °C. Plates were washed six times with PBST and then blocked with 5 % skim milk in PBST for 2 h at 37°C, followed by the addition of 100μl of swab contents diluted in PBS to each well and incubated for 1 h at 37°C. The washing was repeated after each step. Anti-K88 egg-yolk antibodies (100 μl of 10 μg/ml) were added to the plates and then incubated for 1 h at 37°C. The color was developed by the addition of rabbit anti-chicken alkaline phosphatase conjugated antibody as third antibody and pNPP as substrate. The OD was measured at 405 nm using microplate reader model 450 (BioRad).

3.3.10.5 Electrophoresis and immunoblotting: SDS-PAGE electrophoresis was performed with a 12%-acrylamide mini gels using a discontinuous buffer system described by Laemmli, (1970). About 10 μg of pure K88 fimbriae were loaded per lane. Immediately after the completion of the run, gels were equilibrated in the transfer buffer (25 mM Tris, and 192 mM glycine) for 15 min. Proteins were then transferred from the gels onto the nitrocellulose (NC) membranes using a semi-dry trans-blot cell (BioRad). After completion of transfer, the NC membranes were blocked with 3% gelatin in Tris-buffered saline (pH 7.5) for 2 h at 37°C. NC membranes were then probed with mouse serum containing Ab3 and incubated overnight at room temperature. The blots were developed with alkaline phosphatase conjugated goat anti-mouse Ig and nitro blue tetrazolium /5-bromo-4-chloro-3-indolyl phosphate substrate. The reaction was stopped by soaking NC membranes in distilled water.

3.4 RESULTS

3.4.1 Production and purification of anti-K88 monoclonal antibodies

Three fusions which resulted in more than 500 hybridomas were performed to produce mAbs against *E. coli* K88 fimbriae, the majority of which were found to secrete IgM mAbs specific to *E. coli* K88 fimbrial protein. Three clones, two secreting IgM and one secreting IgG2b were chosen and injected into pristane-primed Balb/c mice to obtain ascites fluid. Ascites fluid was clarified and purified by FPLC gel filtration for IgM or by protein-A Sepharose chromatography for IgG2b. Figure 3.1 shows a typical elution profile for mouse IgM from a Superose 12HR 10/30 gel filtration column. When tested for IgM activity, tubes 12 and 13 (shaded peak) were positive while all others tested negative. Samples from tubes 12 and 13 and crude serum as a control were further tested for purity by SDS-PAGE electrophoresis (data not shown). It appeared that the IgM was pure with minor contaminants that might have co-eluted with it. Mouse IgG2b mAb when purified on protein-A Sepharose (Figure3.2) was shown to yield only a single band when subjected to non-reducing electrophoresis (data not shown) .

3.4.2 Fractionation of polyclonal antibodies and production of F(ab')$_2$ fragments

Serum from rabbits immunized with pure K88 fimbriae exhibiting high titer was pooled and fractionated into IgG1, IgG2a, and IgG2b on the protein-A Sepharose column. Serum was applied to the column at pH 8.0 washed and eluted with buffers of increasing acidity (Figure3.3). As indicated, IgG1 (A), IgG2a (B) and IgG 2b (C) were resolved into distinct peaks with the most predominant isotype being IgG2a. The individual protein peaks were collected following each pH change. Fractions from each peak were pooled and analyzed for purity by SDS-PAGE

Figure 3.1: Elution pattern of mouse IgM monoclonal from a Superose® 12 HR 10/30 gel filtration column (30 X 1.5 cm). Serum (0.5 ml) was applied to the column that was equilibrated with 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7.2). The flow rate was 0.4 ml/min. Fractions were monitored for protein at A_{280}. ELISA was used for testing the activity of IgM while its purity was tested by SDS-PAGE electrophoresis. IgM in the shaded area was pure and used for production of Ab2.

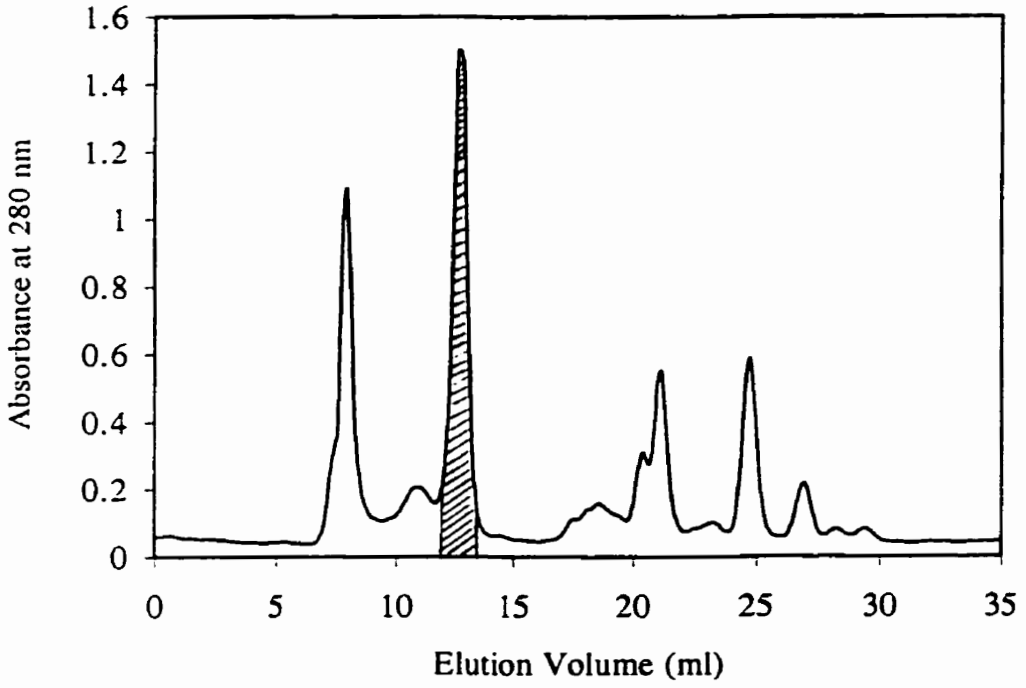

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 280 nm
0
5
10
15
20
25
30
35
Elution Volume (ml)

Figure 3.2: Elution of mouse IgG2b monoclonal from protein A-Sepharose. Ascites fluid (3 ml) was mixed with 2 ml of 0.1 M phosphate buffer (pH 8.0) and adsorbed to the protein A-Sepharose column (10 X 1.5 cm) that was equilibrated with 0.1 M phosphate buffer (pH 8.0). It was eluted with 0.1 M citrate buffer (pH 3.5) at a flow rate of 0.5 ml/min. Fractions were tested for protein content using the Pierce protein assay (BCA). Shaded peak containing the IgG2b activity was pooled and used for Ab2 production.

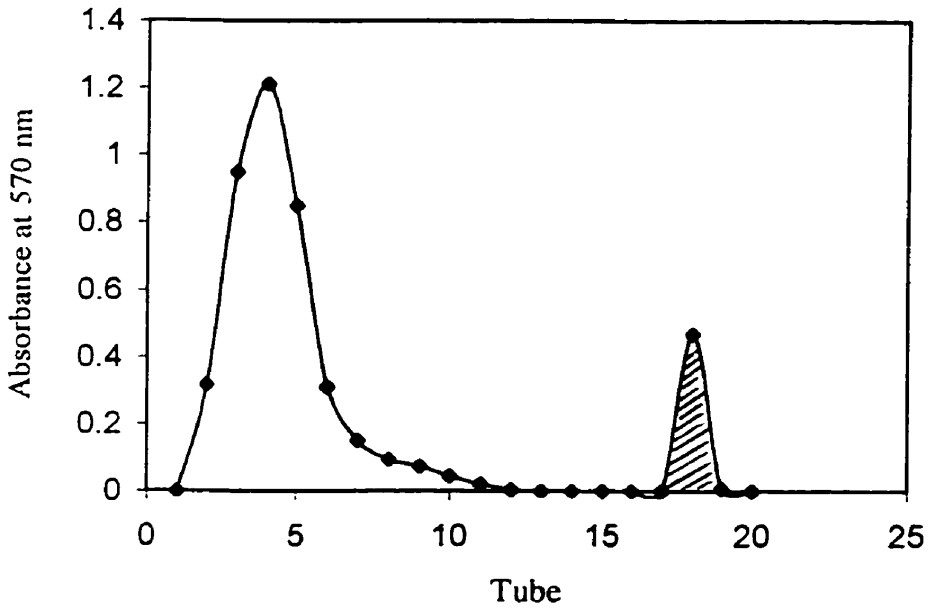
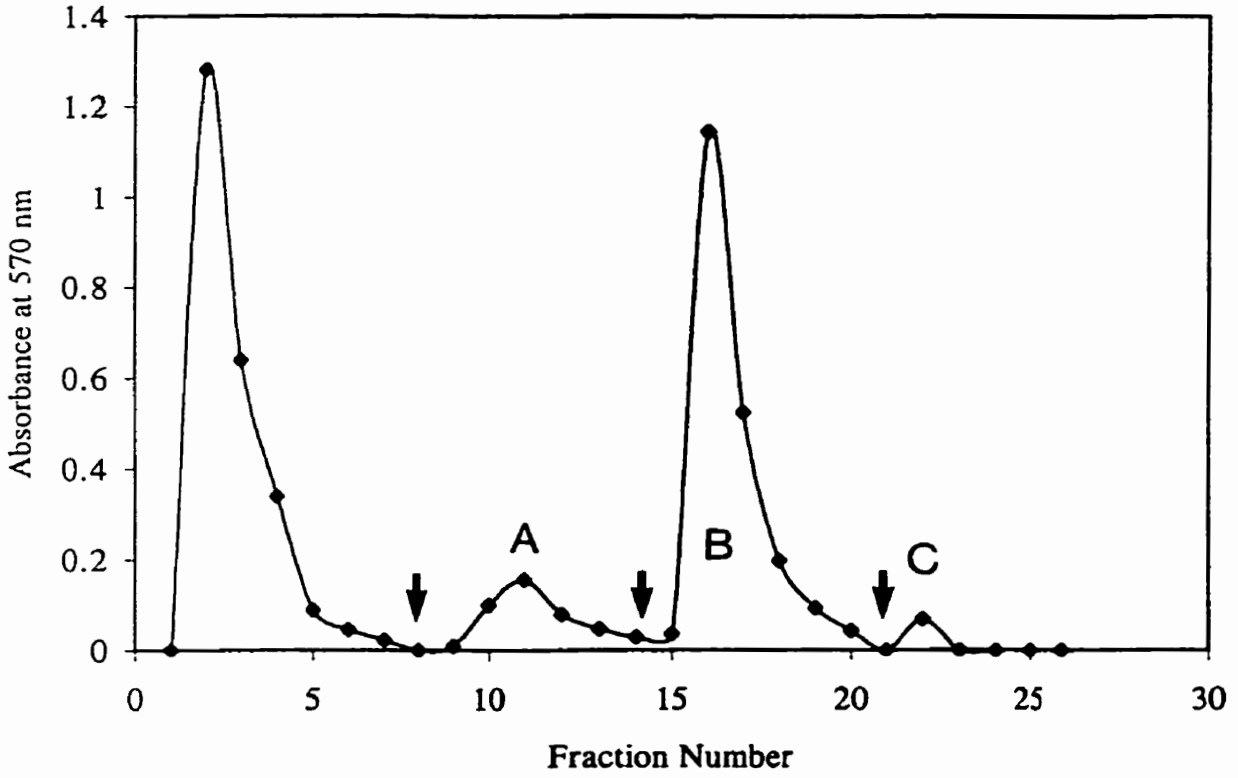

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 570 nm
0
5
10
15
20
25
Tube

Figure 3. 3: Elution of rabbit immunoglobulins from protein A-Sepharose. Serum (3 ml) was mixed with 2 ml of 0.1 M phosphate buffer (pH 8.0) and applied onto the column (10 X1.5 cm). The column was then washed with the same buffer and Igs were then eluted at pH 6.0 for IgG1 (peak A), pH 4.5 for IgG2a (peak B) and pH 3.5 for IgG2b (peak C). The flow rate was 0.5 ml/min. Fractions were tested for protein content using the Pierce protein assay (BCA) and for purity by SDS-PAGE. Peak B and its $F(ab')_2$ were used for production of Ab2.


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 570 nm
A
B
C
0
5
10
15
20
25
30
Fraction Number

electrophoresis. Each fraction exhibited only two bands corresponding to the heavy and light chain with no contaminants (data not shown). The IgG activity of peaks A, B and C as determined by indirect non-competitive ELISA also followed the same pattern as obtained for the protein elution profile. The predominant IgG2a isoform (peak B) was used in subsequent studies. Part of the IgG2a fraction was subjected to pepsin digestion for 8 h in acetate buffer (pH 4.2), purified by FPLC gel filtration chromatography and tested for activity by ELISA. Samples from tubes 11 and 12 (Figure 3. 4, shaded peak) gave a positive ELISA while samples from all other tubes were negative. Native and SDS-PAGE electrophoresis of the pooled $F(ab')_2$ fraction (tubes 11 and 12) indicated that it was pure (Figure3.5).

3.4.3 Specificity of Ab1 for K88 fimbriae

Immunoblotting was carried out to assess the specificity of the Ab1 preparations that were utilized to generate anti-idiotypic antibodies. Fibmrial protein (10 μg /lane) were subjected to SDS-PAGE and immunoblotting and were probed with Ab1 preparations. Monoclonal antibodies (mAb1) were found to react with only one band corresponding to 28 kDa which is the molecular weight of the K88 fimbriae (Jacobs and de Graaf, 1985). The polyclonal antibodies mainly recognized the 28 kDa fimbrial protein. They also reacted to a minor degree with other protein bands that were not detected by silver staining (data not shown). This may be attributed to the presence of trace amounts of proteins other than fimbriae. These data suggests that mAb contained only antibodies against fimbriae whereas the pAb1 also recognized, to a minor degree, proteins other than fimbriae or breakdown products of fimbriae that were antigenically altered, and therefore not detected by mAb1 due to their narrow specificity.

Figure 3.4: Elution pattern of $F(ab')_2$ fragments from the Superose® 12 HR 10/30 gel filtration column. Digested $F(ab')_2$ (0.5 ml) was applied to the column that was equilibrated with 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7.2). The flow rate was 0.4 ml/min. Fractions were monitored for protein at A_{280}. Protein from the shaded area tested positive for IgG activity as determined by ELISA and was shown to be pure as determined by native and SDS-PAGE electrophoresis. It was used for production of Ab2.

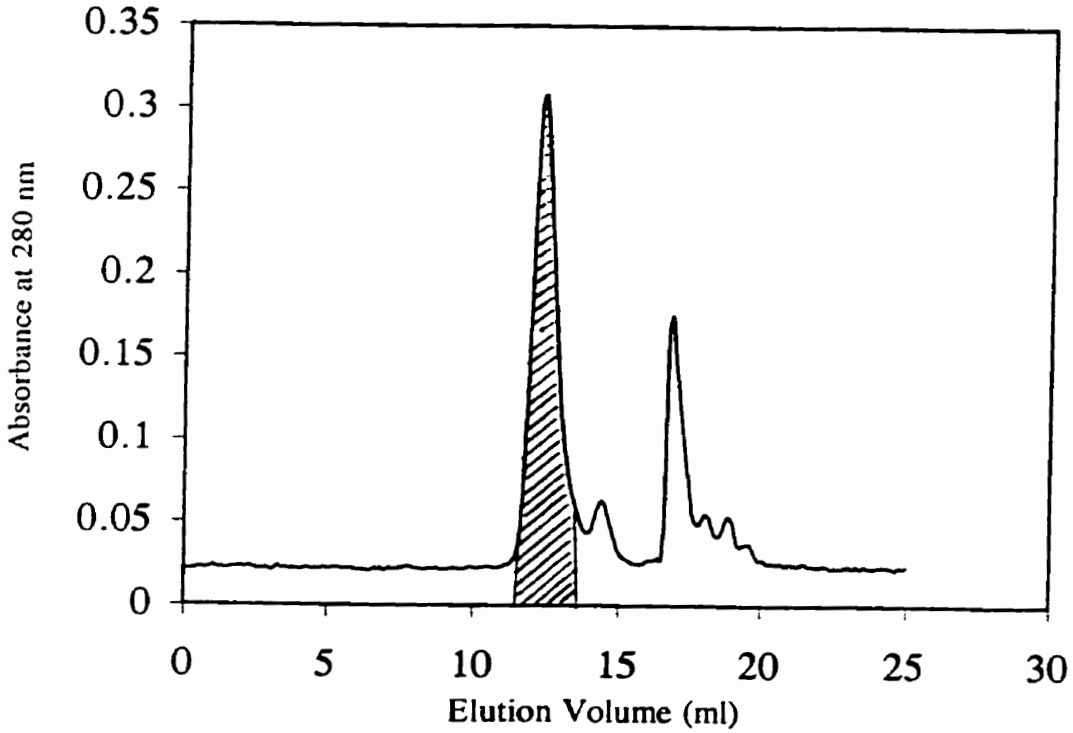
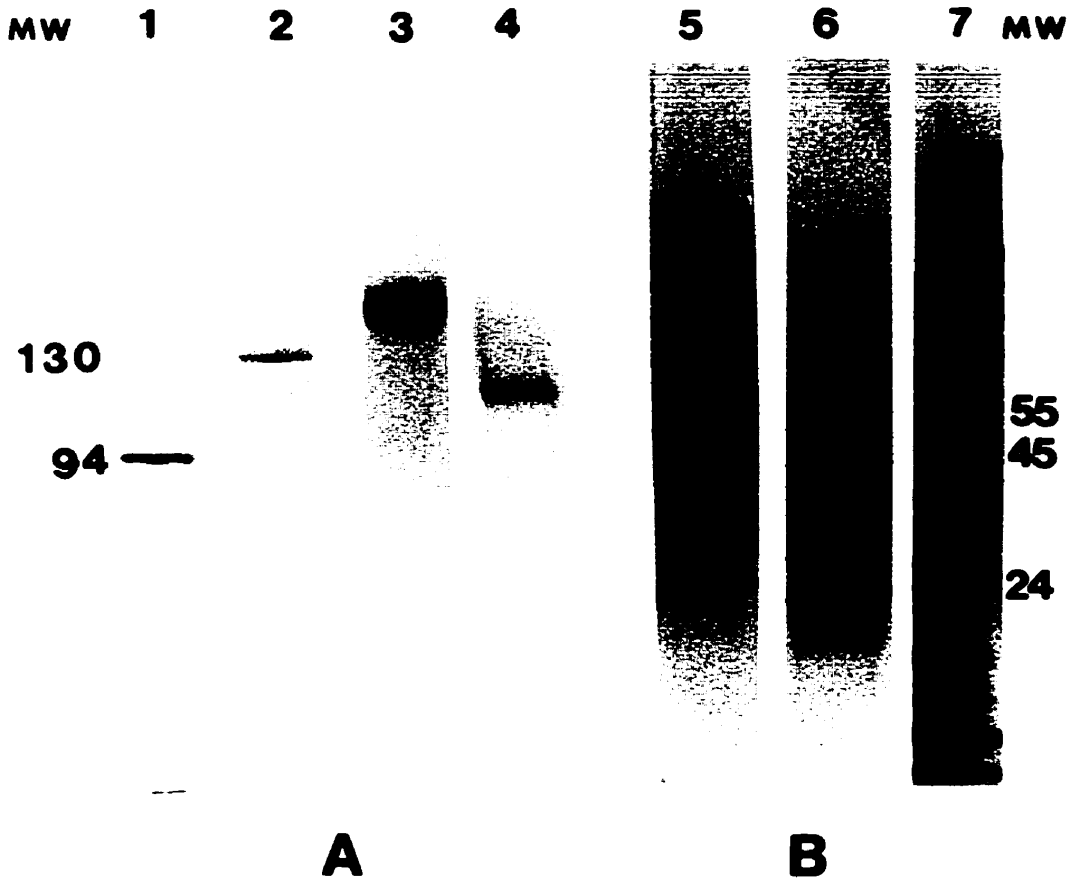

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance at 280 nm
0
5
10
15
20
25
30
Elution Volume (ml)

Figure 3.5: SDS-PAGE electrophoresis of purified rabbit IgG2a and its $F(ab')_2$ fragments under non-reducing (lanes 1-4, A) and reducing (lanes 5-7, B) conditions. Molecular weight standards in lanes 1, 2 and 7 were phosphorylase (MW 94 kDA), β-galactosidase (MW 130 kDa) and a mixture of Sigma markers (MW 6.5 to 206 Kda), respectively. Rabbit IgG2a (lanes 3 and 5) and its $F(ab')_2$ fragments (lanes 4 and 6) were analyzed.


MW
1
2
3
4
5
6
7
MW
130
94
55
45
24
A
B

3.4.4 Production, purification and characterization of anti-idiotypic antibodies

Titration curves were obtained for all the Ab1 preparations used for chicken immunization. Figure 3.6 compares the avidity of the polyclonal and monoclonal antibodies for K88 fimbriae. It is clear from the results that the polyclonal antibodies (upper three curves) exhibited considerably higher avidity for the K88 antigen than monoclonal antibodies (lower three curves). It is worthwhile to notice that at an antibody concentration of 0.05 ng/well the polyclonal antibodies still recognized K88 fimbriae giving an OD of 0.8 for IgG2a and IgG and 0.35 for $F(ab')_2$ while the monoclonals gave the same OD at a dilution of 0.78 μg/well for IgM and 6 μg/well for IgG2b. The corresponding difference in concentration of the two mAb1 was approximately 10,000 and 100,000 greater than those of the pAb1. Among the pAb1, IgG2a and IgG had much higher avidity than the $F(ab')_2$. Equal amounts of these antibodies were used for chicken immunization to produce Ab2. After five injections, all chicken showed an anti-idiotypic titer of > 2,000,000 compared to 500 for the pre-immune sera. Anti-idiotypic antibodies were then purified using the FPLC 12HR 10/30 Superose gel filtration column using same conditions as for IgM.

The anti-idiotypic antibodies were in part characterized by indirect competitive ELISA. It was used to demonstrate the presence of anti-idotypic antibodies that bear an internal image for K88 in chicken serum. In this assay the competition was between a fixed amount of Ab2 and variable amount (serially diluted) of fimbriae (inhibitor) for binding of the corresponding Ab1 (Figure3.7a and b). Anti-idiotypic antibodies produced against pAb1 had at least a two fold higher percent inhibition (*ca.* 28-35 %) than for those produced against mAb1 (*ca.* 6- 15 %). There was no significant difference in percent inhibition within each of the two groups.

Figure 3.6: Reactivities of Ab1 from rabbit: IgG2a (♦), $F(ab')_2$ (■) and IgG (△) or mouse IgG2b (▲), IgM1(*), and IgM2(•) with pure K88 fimbriae. Equal amounts (100μg/ in 200 μl PBS/well) of pure antibodies were serially diluted and tested against K88 fimbriae that was coated onto microtiter plates (1 μg /well) by indirect non-competitive ELISA. Each point represents the average of a triplicate set of analysis.

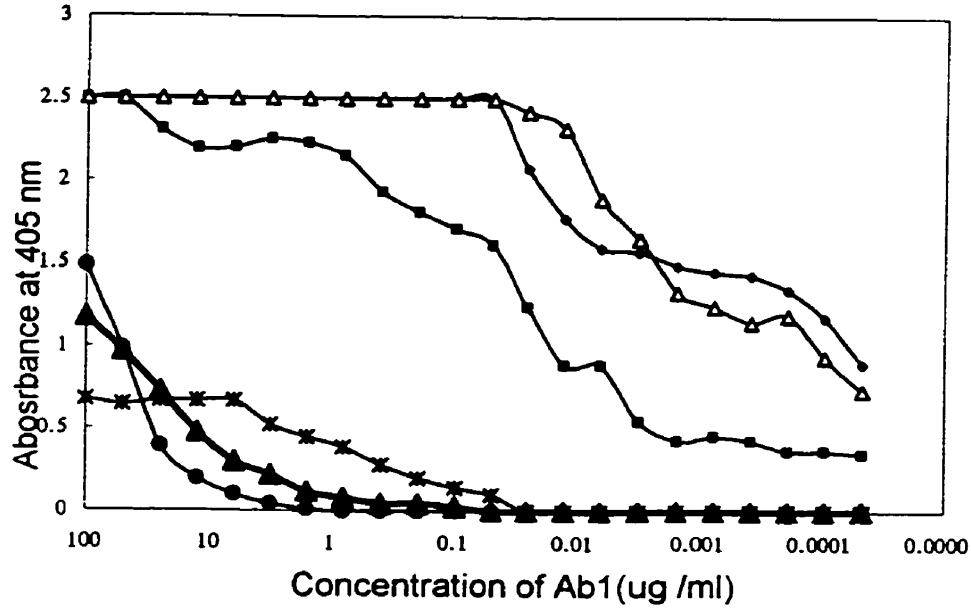
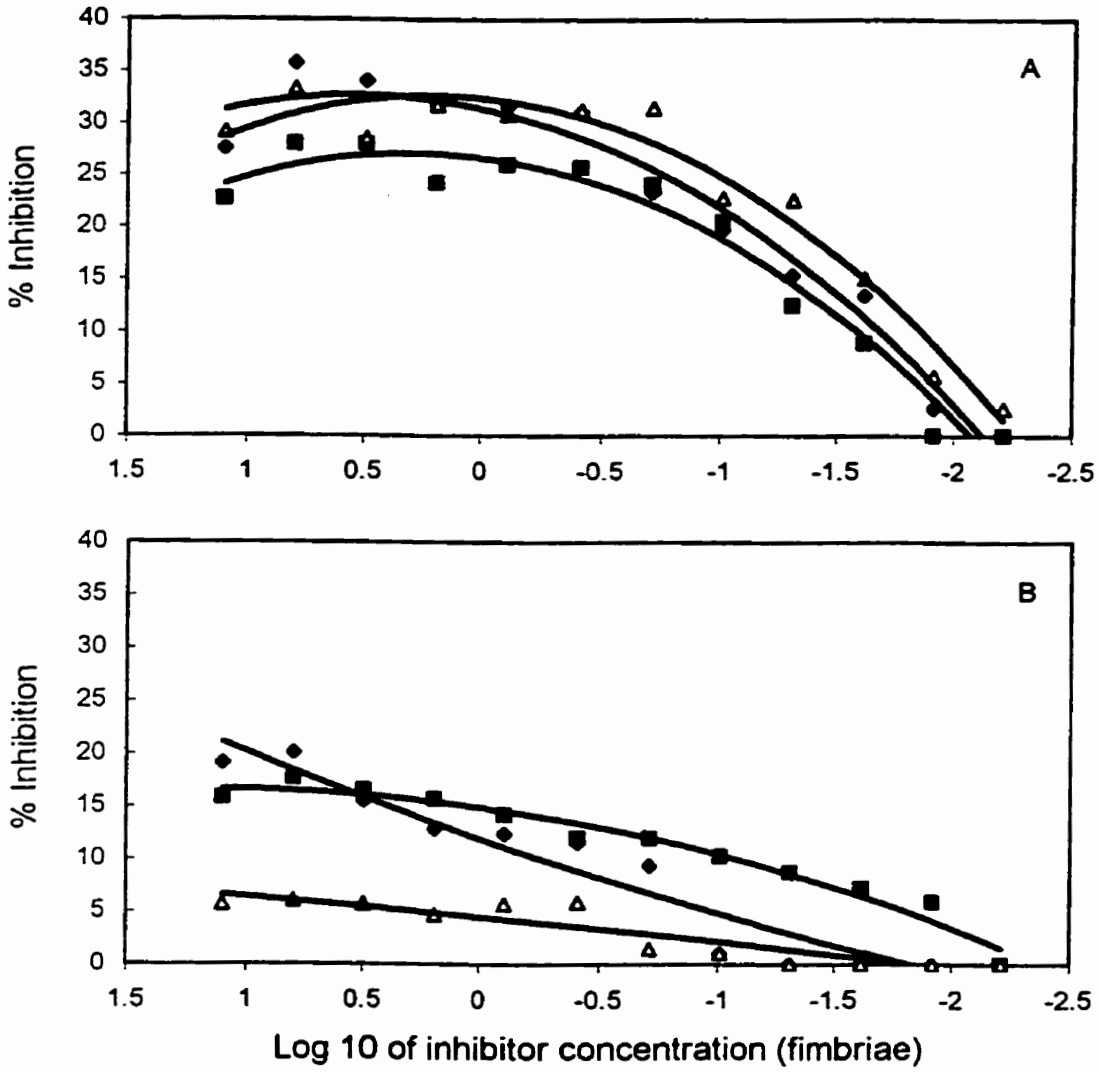

3
2.5
2
1.5
1
0.5
0
Abgsrbance at 405 nm
100
10
1
0.1
0.01
0.001
0.0001
0.00001
Concentration of Ab1(ug /ml)

Figure 3.7a and b: Inhibition of binding of pure K88 fimbriae to Ab1 by anti-idiotypic antibodies (Ab2) as tested by competitive ELISA. In the assay equal amounts (100μl of 10μg/ml/well) of pure Ab1 were coated onto the plates and they were allowed to compete with fixed amounts of pure Ab2 from chicken serum (0.4 μg/well in 50 μl PBS) and variable amounts of K88 fimbriae. Upper frame (A) represents Ab2 produced against rabbit pAb1 [IgG2a (■), $F(ab')_2$ (▵) or IgG (♦)]. Antibodies (Ab2) in the lower frame (B) were produced against mouse mAb1 [IgG2b (▵), IgM1 (♦) or IgM2 (■)]. The best fit lines were obtained using Microsoft Excel. Values are average of a triplicate set of analysis.


A
B
% Inhibition
% Inhibition
40
35
30
25
20
15
10
5
0
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
Log 10 of inhibitor concentration (fimbriae)

3.4.5 Humoral response in mice immunized with anti-idiotypic antibodies

Balb/c mice were immunized with different anti-idiotypic antibodies (Ab2) and with pure K88 fimbriae to determine if the anti-idiotypic antibodies bear an internal image of the original immunogen (K88 fimbriae). The antibody response of mice was evaluated by direct non-competitive ELISA. Figure 8a shows the progress in the production of anti-idiotypic antibodies (Ab3) in mice against chicken Ab2 over 6 wk period. It appeared that anti-idiotypic antibodies produced against all Ab2 antigens gave similar overall results. This indicates that the different anti-idiotypic antibodies from chicken serum (Ab2) produced a similar nonspecific Ab3 response in mouse serum. The ability of the Ab3 in the same serum to react specifically with K88 fimbriae was also tested. The results indicated that the greatest reaction between Ab3 and fimbriae was obtained at six wk when its antigen (chicken Ab2) was produced against rabbit pAb1 (IgG2a, $F(ab')_2$ and IgG). The lowest value for Ab3 was obtained when Ab2 was produced against mAb1 (Figure3.8b). Figure 8b also shows that the degree of reaction of fimbriae with mouse anti-K88 fimbriae (pAb1) was greater than it was with the anti-anti-idiotypic antibodies (Ab3). The titers for K88 fimbriae in the various Ab3 serum from mice were also determined (Figure9a). The absorbance values for serum from mice after 6 wk of immunization, which is a measure of titer, followed a pattern similar to that obtained in Figure3.8b. In these studies all anti-idiotypic preparations gave a similar titer (about 4000) considering an absorbance value of > 0.1 as positive. However, at a dilution of 1:30, sera from mice that were immunized with anti-idiotypic antibodies (Ab2) against $F(ab')_2$ and IgG2a showed an OD of 2.5, followed by sera from mice immunized with anti-IgG Ab2 (OD 2.0). In contrast, sera from mice immunized with chicken Ab2 from the different anti-mAb1 exhibited lower OD values (1.0). A titer curve for mouse pAb1 against K88 fimbriae (Figure3.9b) indicated that

Figure 3.8a and b: Detection of anti-anti-idiotype antibody (Ab3) response in serum of Balb/c mice over a six wk period by indirect non-competitive ELISA. Balb/c mice were immunized with Ab2 that were produced in chicken against anti-K88 rabbit pAb, IgG2a (◇), $F(ab')_2$ (■) and IgG (▲) or mouse monoclonal anti-K88 IgG2b (□), IgM1 (♦), IgM2 (*) antibodies. A) detection of Ab3 response to Ab2 in serum. The plates were coated with a fixed amount of Ab2 (100 μl of 10 μg/ml diluted Ab2/well) and 100 μl of Ab3 diluted 1:2000 were added to each well. B) Detection of Ab3 (diluted 1:30) in response to K88 fimbriae. In this assay fimbriae was coated on the plate (100 μl of 10μg/ml/ well). Figure 8b also shows the antibody response for mice immunized with K88 fimbriae (x). Serum was collected prior to each antigen boost. Other procedures were described in Materials and Methods. Each point represent an average a triplicate set of analysis.

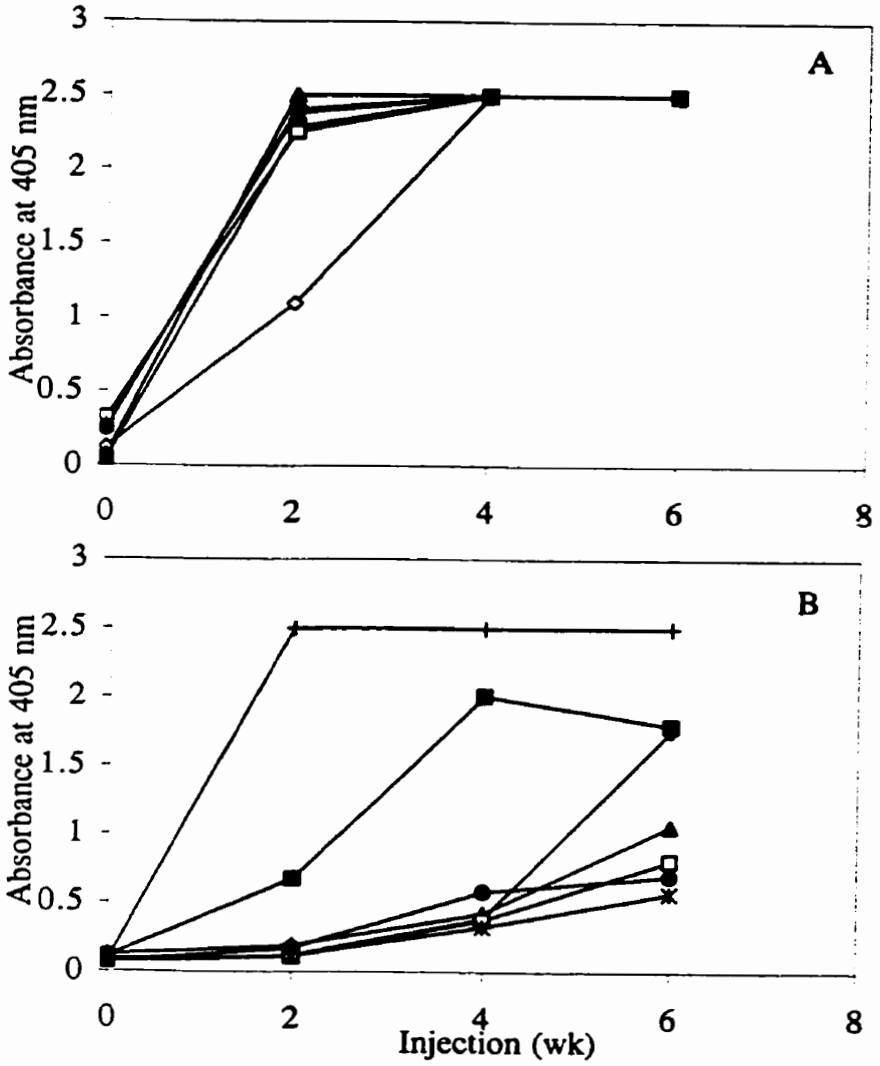
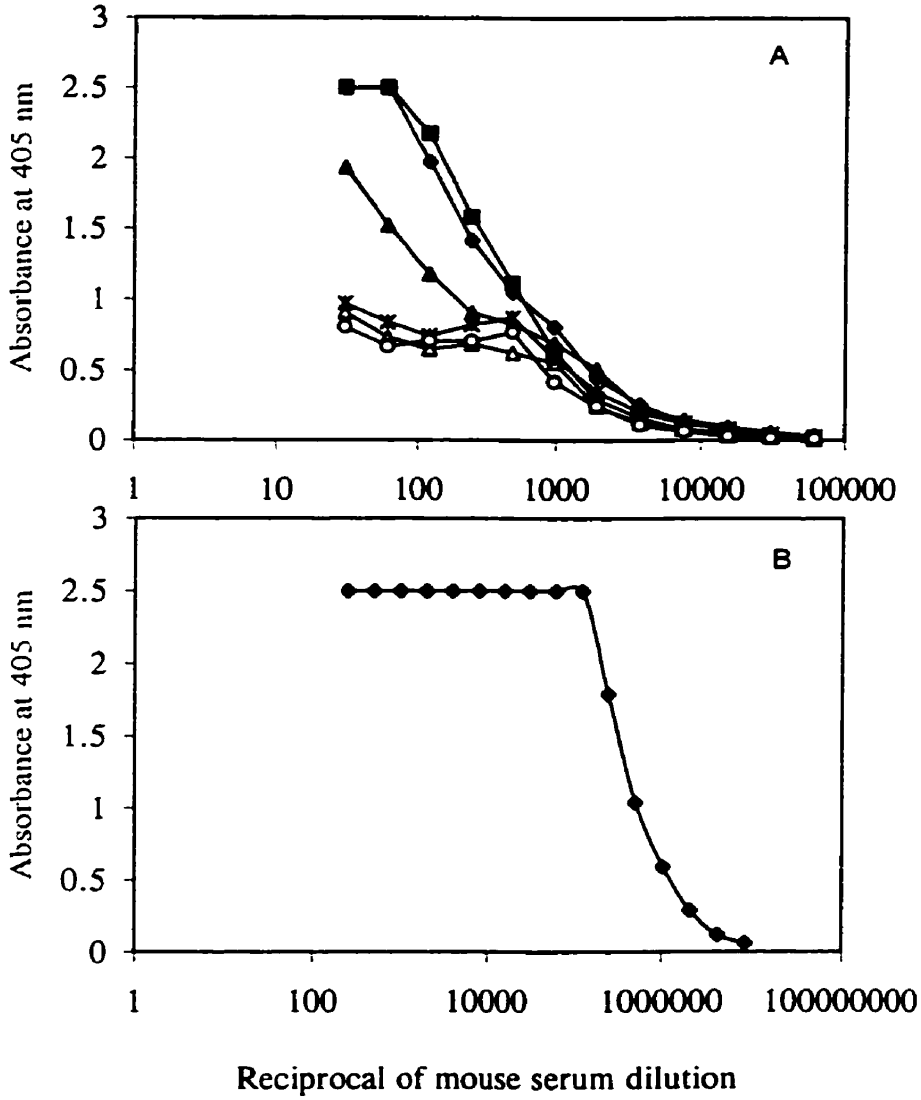

A
B
Absorbance at 405 nm
Absorbance at 405 nm
0
0.5
1
1.5
2
2.5
3
0
2
4
6
8
Injection (wk)

Figure 3.9a and b: Titration curves of serum (Ab3) of mice after 6 wk of immunization as tested by indirect non-competitive ELISA against K88 fimbriae. A) Mice were immunized with chicken Ab2 that was produced against rabbit polyclonal anti-K88 IgG2a (♦), $F(ab')_2$ (□), IgG (▲) or mouse monoclonal anti-K88 IgG2b (△), IgM1 (◇), IgM2 (*). The titer plate wells in this assay were coated with 100 μl of 10 μg/ml pure K88 fimbriae followed by 100 μl of serially diluted Ab3 (initially diluted 1:30) B) Titration curves of serum (1:250 dilution) from mice immunized with K88 fimbriae and tested against the same antigen. Serum was collected prior to each boost.


A
B
Absorbance at 405 nm
Absorbance at 405 nm
3
2.5
2
1.5
1
0.5
0
1
10
100
1000
10000
100000
1
100
10000
1000000
100000000
Reciprocal of mouse serum dilution

the anti-K88 antibody titer in pAb1 was about 20,000 fold greater than that in the highest titer produced in the different Ab3 . In these comparisons the titer was the dilution obtained at half maximum absorption.

3.4.6 Immunoblot analysis of mice serum (Ab3) immunized with various anti-idiotypic antibodies (Ab2)

To further demonstrate the specificity of Ab3 to K88 fimbriae, 10 μl/lane of purified K88 fimbriae were subjected to SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes. The membranes were then probed with mice serum containing Ab3 against the different anti-idiotypic antibodies (Ab2). Serum from all mice had anti K88 anti-anti-idiotypic antibodies that recognized the K88 antigen, while non-immunized serum failed to recognize the K88 fimbriae (Figure3.10). Further, all the Ab3 preparations recognized the K88 fimbriae band without significant difference in the intensity of recognition.

3.4.7 Mouse protection experiment

Protection of vaccinated mice against IP administered *E. coli* K88 was evaluated seven days after the last injection with the Ab2. The protective effects of each Ab2 are shown in Table3.1. The survival rate for all mice that were immunized with Ab2 antibodies was 100% in both experiments 1 and 2. In contrast, the survival rate for non-immunized mice was only 25 % for experiment 1 and 18 % for experiment 2. All immunized and non-immunized mice, except for those immunized with pure K88 antigen, showed signs of infection within 4 h as indicated by a marked decrease in their activity. However, after 24 h these signs disappeared from all immunized mice while the survivors

Figure 3.10: Immunoblott analysis of K88 fimbrial antigen when probed with serum (Ab3) from mice immunized with Ab2 that was obtained from chicken serum produced against anti-rabbit (pAb1) IgG2a, $F(ab')_2$, or IgG (Lane 1,2, 3), respectively. Lanes 4, 5, 6, were chicken Ab2 that were produced against anti-mouse (mAb1) IgG2b, IgM1 or IgM2. Lane 7 was the pre-immune serum.

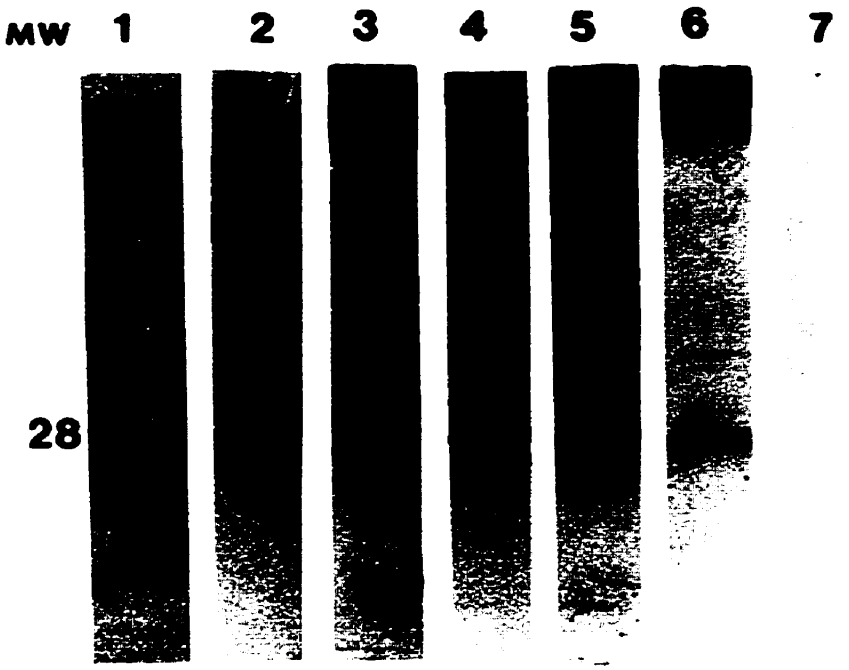

MW
1
2
3
4
5
6
7
28

Table 3.1: Mortality rates of mice immunized with anti-idiotypic antibodies (Ab2) and challenged with *E. coli* K88. (Experiments 1and 2)

Ab2 Treatment[b]	Source of Ab[a]		No of mice per group		Exp. 1			Exp.2		
	Ab1	Ab2	Exp. 1	Exp.2	Disease[c] 4h	24h	Mortality %	Disease 4h	24h	Mortality %
Anti-Fab2	Rabbit	Chicken	2	6	+	-	0	+	-	0
Anti-IgG	Rabbit	Chicken	2	6	+	-	0	+	-	0
Anti-IgG2a	Rabbit	Chicken	NT[d]	6	ND[e]	ND	ND	+	-	0
Anti-IgM1	Mouse	Chicken	2	6	+	-	0	+	-	0
Anti-IgM2	Mouse	Chicken	4	6	+	-	0	+	-	0
Anti-IgG2b	Mouse	Chicken	2	6	+	-	0	+	-	0
K88 fimbriae	-	-	NT	6	ND	ND	ND	-	-	0
No treatment	-	-	4	11	+	+	75(3/4)	+	+	82(9/11)

[a] The rabbit and chicken antibodies are polycolnals while the mice were monoclonals.

[b] In experiment 1 and 2, mice were injected IP with 200 μl and 250 μl, respectively, of 2 X10^8 CFU per mouse of *E. coli* K88. One group of mice were immunized with K88 fimbriae as a positive control while another group was left without any treatment as negative control.

[c] the disease status was considered to be positive (+) in mice where their activity markedly decreased and they stop eating and drinking. Otherwise they were considered to be normal.

[d] This antibody was not tested in experiment 1.

[e] Not determined.

of non-immunized mice continued to show signs of infection for at least one more day.

3.4.8 Passive protection of piglets

Young piglets were orally infected with *E. coli* K88 and some were orally treated with two of the chicken anti-idiotypic antibodies (anti- rabbit IgG2a and anti- rabbit $F(ab')_2$) that had the highest activity as determined by competitive ELISA against K88 fimbriae. The results from this experiment demonstrated that all the piglets challenged with *E. coli* developed diarrhea within 8 h (day 0). This was also apparent from the severe weight loss observed after 24 h of the challenge (Table3.2) which was caused by loss of body fluids. The severity of diarrhea was indicated by a 0-3 scoring system and by testing the presence of *E. coli* K88 in fecal swabs taken daily. After 24 h (day 1) of *E. coli* challenge, fecal scores were the highest (2.9, severe) for the group treated only with *E. coli*, while it was 1.7 (mild) and 1.4 (mild) for (Ab2 treated groups) groups 3 and 4, respectively. The fecal scores decreased dramatically in groups 3 and 4 (within 72 h of the challenge) but increased in the control group with no *E. coli* challenge and no Ab2 treatment. This was attributed to cross-infection as indicated by the presence of *E. coli* in the fecal swabs. No meaningful incidence of diarrhea or fecal scores were obtained from group 2 piglets on days 2 and 3 since only 1 piglet of 8 survived. The fecal scores as indicated in Table 3.2 were consistent with the swab results. After 24 h (day 1), swabs from challenged groups gave an absorbance of about 1.0 which decreased toward the end of study to 0.79 and 0.45 for groups 3 and 4, respectively. The single surviving piglet in group 2 had a high *E. coli* count after day 3 as indicated by the high absorbance obtained by the ELISA assay. In contrast to the antibody treated group, the non-*E. coli* and non-Ab2 treated group had an increasing concentration of *E. coli* in fecal swabs during the time course of the

Table 3.2: Incidence of diarrhea, fecal scores, changes in body weight, presence of *E. coli* K88 in the fecal swabs and mortality of treated and untreated piglets when treated passive with anti-K88 anti-idiotypic antibodies.

	Groups			
	1	2	3	4
No of piglets	13	8	8	8
Treatments				
E. coli[a]	-	+	+	+
Ab2[b]	-	-	+	+
Type of Ab2	-	-	anti-Fab2	anti-IgG2a
Incidence of diarrhea (No)[c]				
Arrival	0	0	0	0
Day 0	0	0	0	0
Day 1	4(13)	7 (8)	6(6)	5(8)
Day 2	9(11)	0	2(4)	1(5)
Day 3	6(11)	0	1(4)	0(4)
Fecal scores[d]				
Arrival	0	0	0	0
Day 0	0	0	0	0
Day 1	0.4	2.9	1.8	1.5
Day 2	1.6	0	1.5	0.5
Day 3	1.2	0	0.8	0
E. coli in fecal swabs (OD)[e]				
Day 0	0.2±0.1	0.2±0.1	0.2±0.1	0.1±0.1
Day 1	0.4±0.4	1.0±0.3	1.1±0.3	0.9±0.4
Day 2	0.9±0.5	1.4±0.0	0.8±0.3	0.8±0.4
Day 3	1.0±0.5	1.9±0.0	0.8±0.5	0.5±0.1
Change in weight (g)/piglet				
After 24 h (losses)	34	541	269	374
After 72 h (gains)	155	248	405	512
No and % of piglets survived	10 (77 %)	1 (12.5 %)	4 (50 %)	4 (50 %)

[a] Groups 2, 3 and 4 were given 5 ml of 10^{10} CFU/ml *E. coli* K88 suspended in saline on day 0 by gastric tubing while group 1 was left without treatment.

[b] Groups 3 and 4 were given chicken Ab2 6 h prior to the bacterial challenge and every 8 h after the challenge until the end of the experiment.

[c] Number of piglets with diarrhea as indicated by the fecal consistency of 1 or more. See [d] for fecal scores..

[d] Fecal scores are the average of fecal consistency: 0, normal; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea.

[e] Fecal swabs were tested for the presence of *E. coli* K88 using sandwich ELISA. Results are average absorbance of swabs taken from each group ± standard deviation. There is a high correlation between absorbance values and the number of CFU (Kim et al, 1999).

experiment. As indicated above this was probably caused by cross-infection from the piglets that were challenged with *E. coli*.

The *E. coli* and non-Ab2 treated group (group 2) showed the highest weight loss after 24 h with the average being 541 g, which was equal to 25 % of body weight before treatment. The weight loss in the two treated groups was much less being 269 g (10 %) and 347 g (17 %) for those treated with anti-$F(ab')_2$ and anti-IgG2a antibodies ,respectively. However, statistical analysis showed no significant difference in the weight lost in these three groups. The weight of the non-challenged control group essentially remained constant during the same period. In contrast, after 72 h, the antibody treated groups recovered more than the lost weight while the single surviving piglet in group 2 (non-antibody treated and *E. coli* challenged group) did not. The mortality rate was the highest (87.5 %) among the group that was challenged with *E. coli* but not treated with Ab2, while it was 50% in groups 3 and 4 (the *E. coli* and Ab treated groups). Unexpectedly the non-challenged group (group 1) started to develop diarrhea after 48 h of when the other groups were challenged, as a result, 3 piglets died (23%). As indicated above this was attributed to cross-infection with *E. coli*.

3.5 Discussion

Antibodies contain idiotypes which are regions located in the antigen binding site. These areas are capable of acting as antigens to produce antibodies that are called anti-idiotypic antibodies (Jerne, 1974). Anti-idiotypic antibodies carry an internal image to the original antigen and can be used as surrogate vaccines. Such vaccines have been shown to induce protective immunity in animals that had never been exposed to the nominal antigen (Beauclair and Khansari 1990; Paque et al, 1990; Percival et al, 1990; Collins et al, 1991; Brossay et al, 1993; Tanaka et al, 1994). *E. coli*

K88 strain is responsible for the majority of diarrhea outbreaks in piglets. The major subunit of the K88 fimbrial antigen is believed to be responsible for the attachment and colonization of *E. coli* to the pig intestine (Smyth, 1994). We have produced mAbs and pAbs for this major subunit as shown by the hemagglutination test and immunoblotting of detergent treated K88 fimbriae (data not shown). These antibodies were used to produce anti-idiotypic antibodies that bear an internal image of this major subunit as the anti-idiotypic antibodies were able to inhibit the binding of pure K88 fimbriae to Ab1 in a concentration dependant manner when tested by a competitive ELISA. These antibodies were distinct from anti-idiotypic preparations that are devoid of this fraction. In addition, the competitive assay developed in this study was able to quantitatively determine the proportion of the anti-idiotypic antibodies that bore an internal image (% inhibition) to the original antigen. Interestingly, the anti-idiotypic antibodies produced against anti-K88 pAb1 had at least twice the percent inhibition (*ca.* 28-35 %) of that of the anti-idiotypic antibodies produced against anti-K88 mAb1 (*ca.* 6-15%). The reason for this difference could be related to the specificity of Ab1 where the monoclonal antibodies, being unispecific, would have recognized only one epitope (10-12 a.a) on the K88 fimbrial major subunit, while the polyclonal antibodies being multispecific, would have contained a battery of antibodies that recognize all epitopes in the fimbrial subunit. Therefore, when both where injected into chicken, polyclonal antibodies would have induced the production of anti-idiotypic antibodies that contain a higher proportion of antibodies bearing an internal image for the K88 antigen than monoclonal antibodies and therefore, were more effective.

In this study we also demonstrated that administration of anti-K88 anti-idiotypic antibodies could induce immunity in mice against subsequent infection with *E. coli* K88. The immunity was manifested by complete protection against an *E. coli* IP challenge of immunized mice (100 %

survival both in experiment 1 and 2, table 3.1), while only 25 and 18 % of un-immunize mice survived the challenge in these experiments. Our results seem to contradict results obtained by Paque et al, (1990). They reported the production of anti-idiotypic antibodies against antibodies that recognize an *E. coli* strain possessing type 1 fimbriae. The anti-idiotypic antibodies were unable to induce protective immunity in mice against an IP challenge with *E. coli*. The reason for the difference may be that in the current study, antibodies (Ab1) were produced against the K88 major subunit which contains the adhesion that recognizes the receptor. Therefore, Ab2 bore an internal image to the K88 antigen, consequently it conferred protection in challenge mice. However, type 1 fimbriae consists of eight subunits (A to H) with only Fim H subunit being responsible for adhesion to receptors. Therefore, anti-idiotypic antibodies produced against antibodies that recognize the major subunit (A), which seems to be immunodominant, were either devoid of or contained only minute amount of anti-idiotypic antibodies that bore an internal image of Fim H subunit and consequently failed to protect mice challenged with the *E. coli*.

It is noteworthy that there was no difference in protection among different anti-idotypic preparations. This indicates that the mice immune system needed only a small amount of anti-idiotyic antibodies bearing an internal image of K88 to provoke the production of protective antibodies against *E. coli* K88 infection. Further, when the serum containing Ab3 obtained from immunized mice was used in immunoblotting against K88 fimbriae, there were no difference in the intensity of the bands recognized by all anti-idiotypic antibody preparations. However, when the serum was taken from mice at 2 wk intervals and tested for the presence of anti-K88 antibodies (Ab3) by direct ELISA, it was clear that serum from mice immunized with anti-idiotypic antibodies produced against anti-K88 pAb1 exhibited much higher Ab3 titers against fimbriae compared to

mice that were immunized with anti-idiotypic antibodies against anti-K88 mAb1. These results were consistent with results obtained with the competitive ELISA.

A second study was undertaken to evaluate the efficacy of two anti-idiotypic preparations in protecting piglets against diarrhea caused by *E. coli* K88. Two anti-idiotypic antibody preparations that exhibited the highest percent inhibition in the competitive ELISA and highest Ab3 titer in the mice protection experiment were selected. It is known that *E. coli* K88 attach to receptors in the intestinal mucus and epithelium layer as a prerequisite step prior to colonization and toxin release. The anti-idiotypic antibodies that bear an internal image to K88 when administered prior to bacterial challenge should block these receptors in analogous manner to the corresponding fimbriae. Thus, the anti-idiotypic antibodies that bear internal images to the K88 would compete with bacteria for binding to K88 receptors and would prevent bacterial colonization so that bacteria will be washed out through normal cleansing mechanisms. In this experiment, egg-yolk containing anti-idiotypic antibodies were administered orally to piglets in order to block the K88 receptors. Partial protection (50% survival rate) was observed in piglets treated with anti-idiotypic antibodies and *E. coli* K88, while the survival rate was only 13.5 % among the groups that were challenged with *E. coli* and did not receive the anti-idiotypic antibodies. This latter group exhibited considerable weight loss due to severe diarrhea while the Ab treated groups did not lose as much weight. The fecal scores for Ab treated groups were half the scores of the corresponding non-Ab treated group. These data imply that the anti-idiotypic antibodies were able to partially block *E. coli* K88 receptors resulting in partial protection against infection with the organism.

There are two reasons that could explain the inability of the anti-idiotypic antibodies to provide complete protection in challenged piglets. These are: i) the proportion of the antibodies that

bear an internal image of K88 fimbriae were not enough to block all the receptors, bearing in mind that a high (5 X 10^{10} CFU) *E. coli* K88 dose was administered to each piglet by gastric tubing and ii) some of the anti-idiotypic antibodies might have been digested or inactivated while passing through the stomach.

Our results appeared to be consistent with results obtained by Jackson et al, (1990). They reported incomplete protection of rats against dental carries when anti-*Streptococcus mutants* anti-idiotypic antibodies were administered orally to rats. The anti-idiotypic antibodies used in this study were polyclonal which means only a certain proportion of the pool were effective. However, Reck et al, (1988) reported a complete protection of monkeys against diarrhea and emetic response induced by *Staphylococcus* enterotxin B. They used a monoclonal anti-idiotypic antibody that mimicked the toxin.

The results of the current study indicates that anti-idiotypic antibodies against *E. coli* K88 fimbriae when administered to mice were able to induce active immunity in mice that completely protected them against an IP challenge with *E. coli* K88. The same anti-idiotypic antibodies when used to provide passive immunity against an oral challenge of *E. coli* K88 also provide protection but it was only partial. The protective effect with anti-idiotypic antibodies appeared to be greater when polyclonals compared to monoclonals were used as the source of idiotype antibodies. Also the protective effect of the polyclonal antibodies in mice obtained with the anti-idiotypic antibodies was considerably less than that obtained when they were immunized directly with fimbrial antigen. These results demonstrated that anti-idiotypic antibodies can be useful in the control of *E. coli* infection in pigs and mice but that may not be nearly as effective as idiotype antibodies produced directly against the antigen.

4.Manuscript II

Identification of *E. coli* K88 Receptors in Porcine Intestinal Mucus Using Anti-idiotypic Antibodies

4.1 Abstract

Escherichia coli K88 receptors were purified from porcine intestinal mucus by affinity chromatography using a K88 fimbrial antigen attached to Sepharose 4B. Receptor eluate from the column were identified using anti-idiotypic antibodies that bore an internal image to K88. They were produced in chickens against both mouse anti-K88 monoclonal and rabbit anti-K88 polyclonal antibodies. The anti-idiotypic antibodies were tested against purified receptors using an indirect ELISA. Higher absorbance values(1.3, 1.2, and 1.2) were observed for the anti-polyclonal anti-idiotypic antibodies than those for anti-monoclonal anti-idiotypic antibodies(0.95, 0.81, and 0.56). Analysis of purified receptors by SDS-PAGE electrophoresis and immunoblotting with K88 fimbriae revealed several bands corresponding to 40, 45, 50 and 70 kDa. However, the anti-polyclonal anti-idiotypic antibodies recognized only the 70 kDa protein while the anti-monoclonal anti-idiotypic antibodies failed to recognize any of these proteins. Sugar staining suggested that only the 45 kDa protein contained a sugar moiety. This study indicated that a 70 kDa protein in the intestinal mucus of pigs may be a dominant receptor for K88 and demonstrated that anti-idiotypic antibodies are useful tools for receptor identification.

4.2 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains bearing K88 fimbrial antigen are frequently a causative agent of diarrheal outbreaks in piglets (Laux et al, 1986; Metcalf et al, 1991; Erickson et al, 1992; Blomberg et al, 1993). The first and most critical step in initiating an infection by many pathogens is the recognition of specific host cell receptors (Thaker et al, 1994). The binding of *E. coli* through its fimbrial antigen to the receptors in the intestine allows it to resist and circumvent flushing and cleansing mechanisms; therefore, enabling it to colonize the intestine and eventually cause disease (Klemm, 1985; Erickson et al, 1992; Payne et al 1993; Gyles, 1993; Nystrom, 1995; Erickson et al, 1997). Studies on the identification and characterization of *E. coli*-K88 receptors have been reported by several groups over the past two decades. Staley and Wilson, (1983,1984) identified 23 and 32 kDa receptors from porcine intestinal brush border cells. Metcalf et al, (1991) used electrophoresis and immunoblotting to identify K88 receptors in the intestinal mucus. A 42 kDa receptor was identified and found to be a glycoprotein. Latter, Erickson et al, (1992, 1994) used preparative gel electrophoresis to identify, excise and elute putative K88ac receptors from porcine bush border. Two receptors of MW 210 and 240 kDa were identified using this method, and were characterized by gas chromatography and found to be mucin type sialoglycoproteins. In addition, Willemsen, (1993) showed that porcine intestinal mucus contained three glycoprotein receptors of MW 25, 35 and 60 kDa that bound to K88 ab, ac and ad. Further studies conducted by Blombeg et al, (1993) proved the presence of a glycolipid receptor in porcine intestinal mucus which appeared to be a galactosylceramide. Recently, Fang et al, (1999) developed an affinity chromatography method for isolation of a K88ac receptor. They identified 26 and 41 kDa fimbrial binding proteins using immuoblotting, however, no further studies were conducted to reveal their identity. These

receptors were identified using gel electrophoresis followed by immunoblotting using biotinylated K88 fimbrial antigen or radio-labeled *E. coli* K88 cells.

Anti-idiotypic antibodies (Ab2) proved to be exceptional tools for identification of cell surface receptors (Gaulton et al, 1985). Their use as probes for cell surface receptors was first reported by Sege and Peterson, (1978). They identified receptors for retinol binding protein (RBP) and insulin using anti-idiotypic antibodies that mimic RBP and insulin. After this report, several other receptors were identified using anti-idiotypic antibodies including a reovirus cell surface receptor(Noseworthy et al, 1983), a receptor for atrial natriuretic factor (ANF) (Chua et al, 1989),a cellular receptor for bovine herpes virus 1(Thaker et al, 1994) and many other receptors. However, there are no reports on the use of anti-idiotypic antibodies to identify receptors for any intestinal pathogen. Therefore, the objectives of this paper were to isolate the putative porcine intestinal mucus receptors using affinity chromatography and to use anti-idiotypic antibodies that mimic K88 fimbrial antigen to identify them. Carbohydrate staining was conducted to partially ascertain their composition.

4.3 Materials and Methods

4.3.1 Materials

Polyacrylamide mini gels and nitrocellulose membranes (0.45μm) were purchased from Bio-Rad (Hercules CA, USA). Alkaline phosphatase conjugated rabbit anti-chicken and goat anti-rabbit were from Jackson Immunochemicals (Missisauga, ON). Cyanogen bromide activated Sepharose, 5-bromo-4-chloro-3-indolyl phosphate ρ-toluidine salt (BCIP) and ρ-nitro blue tetrazolim chloride (NBT) were from Sigma-Aldrich Chemical Co.(St. Louis, MO). Falcon microtest III polyvinyl

chloride flexible 96-well assay plates were obtained from Fisher Scientific Ltd. (Nepean , ON) and Bicinchoninic Acid (BCA) protein assay kit was from Pierce (Rockford, IL). All other chemicals and reagents were of analytical grade.

4.3.2 Preparation of E. coli K88 fimbriae

K88 fimbrial protein was extracted and purified as described earlier in Manuscript I. The method was based on a modification of the procedure by Erickson et al, (1992).

4.3.3 Immobilization of K88 fimbriae to Sepharose 4B

The immobilization of K88 fimbriae to cyanogen bromide activated Sepharose 4B was performed according to the manufacturers instructions. Briefly, two grams of Sepharose 4B were washed several times and swelled in cold 1mM HCl for 2 h. The beads were then washed with 5-10 column volumes of distilled water and 0.1 M $NaHCO_3$ containing 0.5 M NaCl (coupling buffer, pH 8.5), and resuspended in the same buffer. Twenty five mg of highly purified fimbrial K88 protein suspended in the coupling buffer were mixed gently with swelled beads in an end to end mixer at 4°C for 16 h. Uncoupled material was removed by washing with the coupling buffer. Unreacted groups on the beads were blocked with 0.2 M glycine (pH 8.0) for 2 h at room temperature. The beads were finally washed with coupling buffer and packed in a 10 X 1.5 cm column.

4.3.4 Mucus extraction

Mucus was isolated from neonatal piglets according to the method of Metcalf et al, (1991) with modification. Briefly, the small intestine was obtained from five 1-h old piglets that were

deprived of colostrum, cut in 20 cm pieces and washed extensively with PBS, (pH 7.4) until the buffer was clear. The cut sections were then split along the mesenteric border and immersed in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid)-Hanks buffer (HH buffer, pH 7.4). A microscopic slide was then used to scrap the mucus into the buffer. Scraps from each segment were pooled and centrifuged twice at 27,000 xg for 15 min at 4°C to remove cells and debris. The supernatant containing the mucus fraction was then analyzed for protein content using the Bicinchoninic Acid (BCA) protein assay kit, tested for residual pig IgG by ELISA and stored in small portions at -80°C until used.

4.3.5 Affinity chromatography

Affinity chromatography was performed using a procedure similar to that of Fang et al, (1999). The packed affinity column was equilibrated with 0.01 M PBS (pH 7.3). About 40 ml of 1 mg/ml mucus diluted in HH buffer (pH7.4) were applied to the column. The column was washed extensively with PBS (pH 7.3) until no absorbance was detected. Receptors where then eluted from the column with 0.2 M glycine (pH 2.3). The A_{280} was determined for each fraction, and tubes contained higher absorbance than the background were pooled. The eluted material was concentrated using either a Speed Vac Savant AS160 (Farmingdale, NY) or by freeze-drying using a VarTis freeze-dryer model Genesis 25 LE (Gardiner, NY).

4.3.6 Production of anti-fimbrial and anti-idiotypic antibodies

Mouse monoclonal (mAb1) and rabbit polyclonal (pAb1) anti-fimbrial antibodies, and chicken anti-idiotypic antibodies that mimicked the K88 fimbrial antigen were produced using the

procedure described in Manuscript I.

4.3.7 Purification of anti-idiotypic antibodies

Chicken serum containing the anti-idiotypic antibodies was fractionated on a Superose 12HR 10/30 gel filtration column equilibrated with 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7) in an FPLC unit (Pharmacia Biotech Inc. Baie durfe, QC). Serum samples were passed through a 0.2 μm filter and 0.5 ml per run was applied onto the column. The chicken IgY was then eluted in the equilibration buffer at a flow rate of 0.4 ml/min. Eluted proteins were monitored at A_{280}. All fractions were tested for activity by ELISA and tubes containing IgY activity were subjected to SDS-PAGE electrophoresis to test their purity. The most pure fractions were pooled and concentrated by precipitation with 25% saturation ammonium sulphate, diluted in PBS to a concentration of 2 mg/ml protein and stored at -80 °C until used.

4.3.8 Detection of putative K88 receptors using anti-idiotypic antibodies by ELISA

Mucus that was extracted from porcine intestine and purified by affinity chromatography was tested for the presence of K88 receptors by ELISA. Microtiter plates were coated with 100μl purified mucus (2 μl/well in 0.1 M carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The contents were then removed and plates were washed 6 times with PBST . The non-specific sites in the wells were blocked with 5% skim milk in PBST and plates were incubated for 2 h at 37 °C. FPLC purified anti-idiotypic antibodies (1μg/well in PBST) were added to each well and incubated for 1.5 h at 37 °C. After 6 washings with PBST, 100 μl of rabbit anti-chicken alkaline phosphatase conjugate antibody diluted in PBST (1:5000) was added to each well and incubated for 1 h at 37 °C.

Plates were then washed 6 times (PBST) followed by the addition of 100 μl/well of pNPP substrate and incubated for 1 h at 37°C. Optical density of the wells was determined at 405 nm using a microplate reader (Bio-Rad Laboratories Model 450).

4.3.9 Polyacrylamide gel electrophoresis

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis was performed on a 12% separating and 4% stacking mini-gels using the discontinuous buffer system described by Laemmli, (1970) in a BioRad mini-gel electrophoresis unit (BioRad). Samples (10 μl) of purified receptors or FPLC purified antibodies were mixed with equal volume of treatment buffer (0.125 M Tris base, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8), boiled for 5 min and loaded onto the gel. Separation was carried out at constant voltage (200 volt) for 45 min. Gels were either stained with silver staining or Coomassie Brilliant Blue R-250 or used immediately for immunoblotting.

4.3.10 Immunoblotting

Immunoblotting was performed according to the method of Davis et al, (1994). Briefly, electrophoresed gels were equilibrated in the transfer buffer (25 mM Tris and 192 mM glycine) for 15 min. Putative receptors were transferred onto nitrocellulose using a trans-blot semi-dry transfer cell (Bio-Rad, Hercules, CA) for 30 min at constant voltage (15 volt). The membranes were blocked with 3% gelatin in TBS (20 mM Tris base, 500 mM NaCl, pH 7.5) for 1 at 37°C. After 3 washings with TBST, membranes were incubated overnight at room temperature with either anti-idiotypic antibodies diluted 1:250 in TBST buffer containing 1% gelatin or with pure K88 fimbriae (100 μg/ml) diluted in the same buffer. The membranes previously incubated with anti-idiotypic

antibodies were washed three times with PBST and developed using alkaline phosphatase anti-chicken immunoglobulins and a mixture of 33 μl of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and 330 μl of nitro blue tetrazolium chloride (NBT) per 10 ml of substrate buffer (100 mM M Tris base, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5). While the membranes incubated with the K88 fimbriae were additionally incubated with rabbit anti-K88 and developed as for the other membranes.

4.3.11 Carbohydrate staining

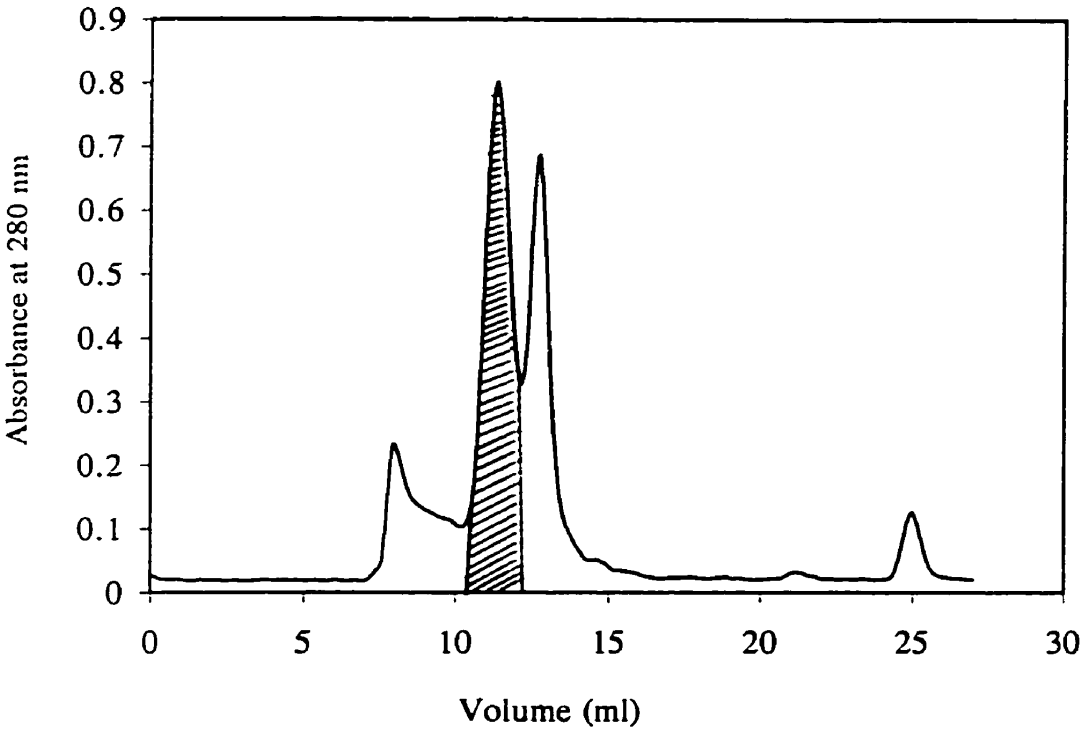
Isolated receptors were stained for carbohydrate contents as described by Kondo et al,(1991). Animals were cared for in accordance with the guidelines established by the Canadian Council on Animal Care (CCAC, 1980).

4.4 Results

4.4.1 Purification of anti-idiotypic antibodies

Anti-idiotypic antibodies (Ab2) produced in chicken against mouse monoclonal and rabbit polyclonal anti-K88 antibodies were used to identify *E. coli* K88 receptors inporcine intestinal mucus. Chicken serum was purified by gel filtration using fast-protein liquid chromatography (FPLC). Figure 4.1. shows a typical elution profile for chicken IgY using Superose 12 HR 10/30 gel filtration column. Samples from tubes 10, 11 and 12 (second peak) tested positive for the presence of IgY activity while other fractions tested negative. Samples from tubes 10-12 and crude serum, as a control were subjected to SDS-PAGE electrophoresis and stained with Coomassie

Figure 4. 1: Elution profile of chicken IgY from Superose® 12 HR 10/30 column. Serum (0.5 ml) was applied to the column that was equilibrated with 0.05 M phosphate buffer containing 0.15 M NaCl (pH 7.2). The flow rate was 0.4 ml/min. Fractions were collected and monitored for protein at A_{280}. Protein from the shaded area was tested for activity by ELISA and for purity by SDS-PAGE electrophoresis, and used for subsequent studies.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 280 nm
0
5
10
15
20
25
30
Volume (ml)

Brilliant Blue R-250. These fractions (tubes 10-12) contained two major bands, a 66 kDa band corresponding to the heavy chain of IgY and a 20 kDa band corresponding to its light chain (Figure 4.2). However, samples from tubes 10 and 12 (lanes 3 and 5) appeared to be contaminated with some minor proteins while these in tube 11 (lane 4) appeared to be the purest with one minor contaminant. Antibodies from tube 11 were used in the subsequent experiments.

4.4.2 Purification of K88 adhesion receptors

In this study, affinity chromatography using pure K88 fimbriae immobilized to 4B Sepharose was used to isolate the K88 putative receptors. Porcine intestinal mucus was collected from piglets deprived of colostrum to ensure the absence of anti-K88 antibodies that might interfere with the purification process and was subjected to K88 affinity chromatography. Fractions were collected and the A_{280} of each fraction was determined (Figure 4.3). ELISA demonstrated that a single peak (tubes 17-23, shaded area) contained the receptor activity.

4.4.3 ELISA for monitoring the binding of anti-idiotypic antibodies to affinity purified receptors

The ability of different Ab2 to recognize the affinity purified receptors was determined using indirect non-competitive ELISA (Table 4.1). The ELISA results showed that Ab2 produced against anti-K88 polyclonal antibodies (pAb1) exhibited higher absorbance than that obtained with Ab2 produced against anti-K88 monoclonal antibodies (mAb1) even though the same quantity of purified Ab2 was added to each well. It appeared that the lowest absorbance was recorded for the Ab2 that was produced against mouse IgM1 mAb while similar absorbance values were obtained among the different Ab2 that were prepared against pAb1.

Figure 4. 2: SDS-PAGE electrophoresis of purified chicken serum. Samples (10 μg/lane) were applied to a 12 % polyacrylamide slab gel using discontinuous buffer system for 45 min at 200 volt and stained with Coomassie Brilliant Blue. Lane 1, molecular weight standards; lane 2, crude serum; and lanes 3, 4, 5 eluates from tubes 10, 11, and 12 (peak 2).

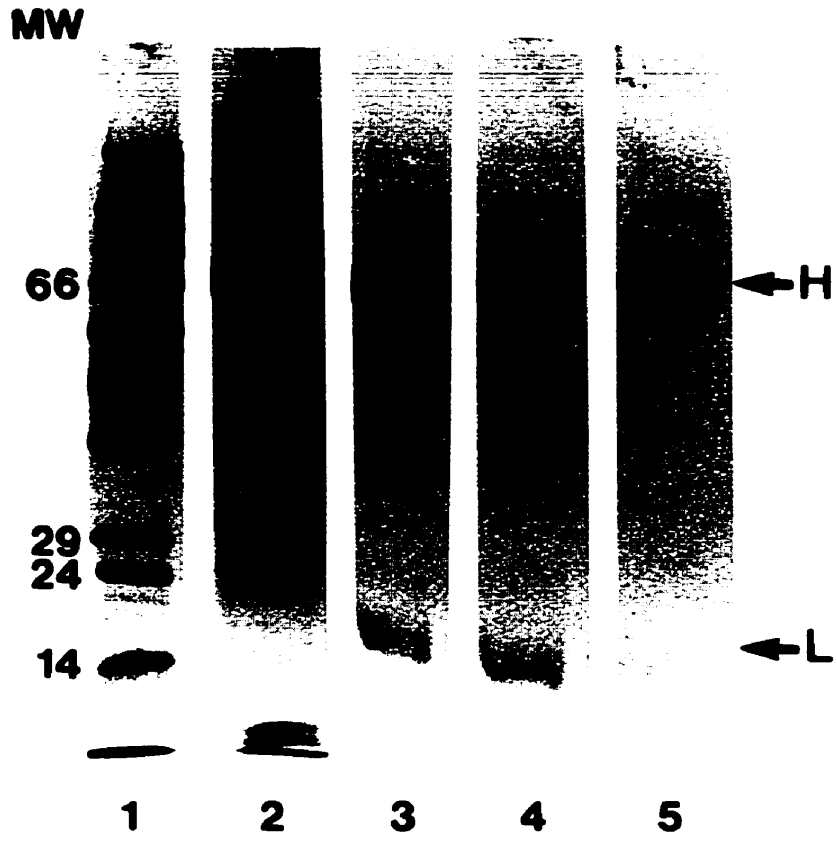

MW
66
29
24
14
H
L
1
2
3
4
5

Figure 4. 3: Elution profile of porcine intestinal receptors from the Sepharose 4B-K88 fimbrial column. A total of 40 ml mucus diluted in HH buffer was applied to the column that was equilibrated with PBS (pH 7.3). The flow rate was 0.5 ml/min and fractions of 1 ml were collected. The elution was monitored at A_{280} and eluate from tubes with absorbance values higher than the background were pooled and used for further characterization. Shaded area contained the receptor activity as detected by ELISA.

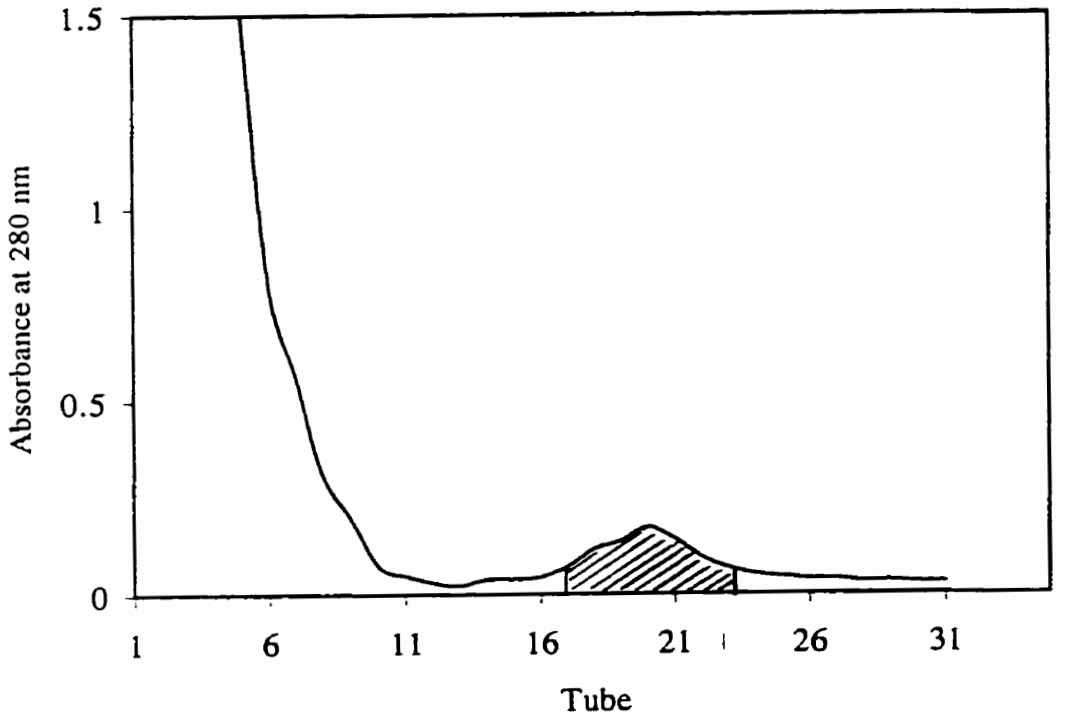

1.5
1
0.5
0
Absorbance at 280 nm
1
6
11
16
21
26
31
Tube

Table 4.1: Binding of *E. coli* K88 Affinity Purified Receptors by Anti-idiotypic Antibodies From Chicken Serum.

Ab 1[a]	Source of Ab1	Type of Ab1	Ab 2[b]	Source of Ab2	Type of Ab2	Average absorbance
IgG2a	rabbit	PAb	anti-IgG2a	chicken	PAb	1.27[c]
IgG2a-Fab2	rabbit	PAb	anti-Fab2	chicken	PAb	1.21
IgG	rabbit	PAb	anti-IgG	chicken	PAb	1.17
IgG2b	mouse	MAb	anti-IgG2b	chicken	PAb	0.95
IgM1	mouse	MAb	anti-IgM1	chicken	PAb	0.56
IgM2	mouse	MAb	anti-IgM2	chicken	PAb	0.81
-	-	-	NIS[d]	chicken	-	0.22

[a] Ab1 were purified anti-fimbriae polyclonal or monoclonal antibody.
[b] Ab2 were purified anti-idiotypic antibodies produced against Ab1
[c] Results are average of triplicate analysis. High absorbance values indicate high binding affinity of Ab2 for the pig mucus receptor.
[d] Non- immunized serum

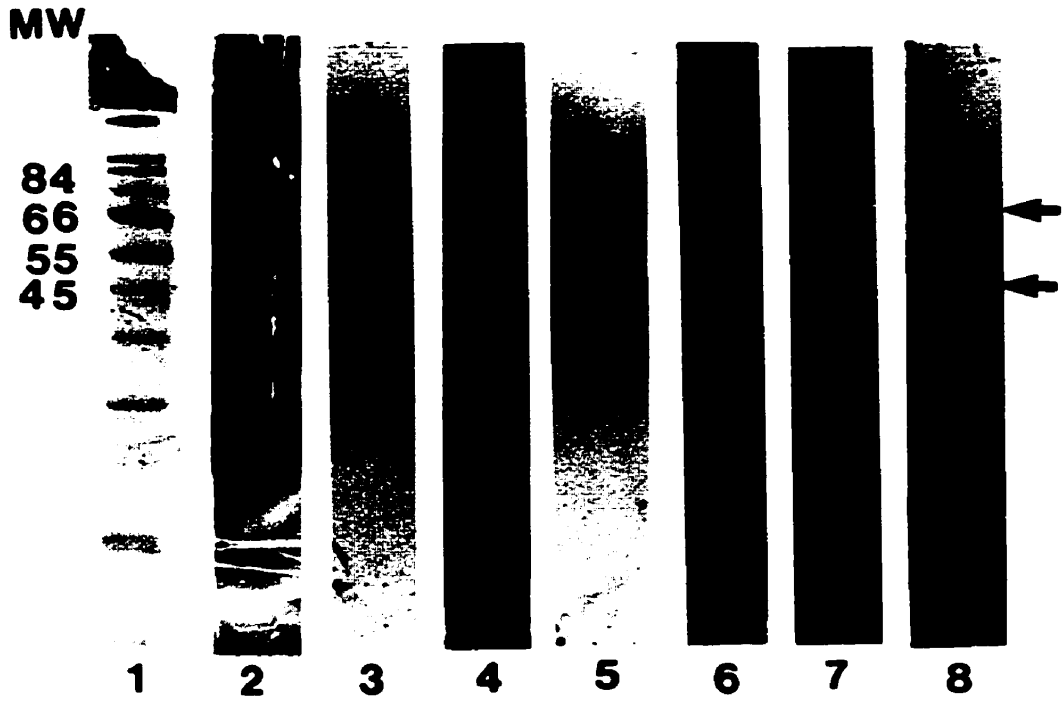
4.4.4 Identification of K88 adhesion binding proteins

Individual proteins that are capable of acting as receptors for *E. coli* K88 fimbriae were identified using gel electrophoresis and immunoblotting. The affinity purified receptors were separated by SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes. Membranes were either probed with K88 fimbriae to identify all K88 binding proteins or were incubated with different anti-idiotypic antibodies. The K88 fimbrial protein bound to 40, 45, 50 and 70 kDa proteins and some other minor proteins having molecular weights between 55 and 66 kDa (Figure 4.4 , lane 4). The presence of these minor proteins may represent breakdown products of higher molecular weight proteins (Metcalf et al, 1991). However, Ab2 produced against polyclonal anti-K88 antibodies recognized only a single protein having a molecular weight corresponding to 70 kDa (Figure 4.4, lanes 3, 4, and 5). In contrast to the ELISA results, Ab2 produced against mouse mAbs did not recognize any of the receptors (Figure 4.4 lane 6).

4.4.5 Nature of identified K88 receptors

Carbohydrate staining of proteins transferred onto nitrocellulose membranes was conducted to determine if the receptor also had a carbohydrate moiety. Only one of the isolated proteins corresponding to a molecular weight of 45 kDa appeared to have a sugar moiety (Figure 4.4 lane 8). However, the 70 kDa receptor identified by Ab2 either did not seem to contain any sugar or it was not detected by carbohydrate staining.

Figure 4. 4: Immunoblot of affinity purified K88 receptors from porcine intestinal mucus. The receptors were separated using SDS-PAGE and probed with anti-idiotypic antibodies, pure K88 fimbriae or stained for sugar contents. Lanes: 1, molecular weight standards; 2, receptor extract probed with K88 fimbriae; 3- 5, receptor probed with anti- rabbit IgG2a, $F(ab')_2$ and IgG anti-idiotypic antibodies, respectively; 6, receptor probed with anti-mouse IgM anti-idiotypic antibody; 7, receptor probed with chicken pre-immune serum; and 8 receptor stained for carbohydrate.


MW
84
66
55
45
1
2
3
4
5
6
7
8

4.5 Discussion

Porcine intestinal mucus contains several proteins and polypeptides that can function as receptors for *E. coli* K88. The isolation and characterization of these receptors is important to understanding their mechanism of infection. This could play a pivotal role in the development of alternatives for prevention of infections caused by this pathogen. However, characterization of receptors is hampered by their minute quantity in mucus, thus, the development of a simple, rapid and quantitative procedure to isolate these receptors in pure form would greatly facilitate further studies on receptors.

Only a few methods for the isolation of K88 receptors have been reported in the literature. Gel filtration is one method that has been frequently used for this purpose (Laux et al, 1986; Metcalf et al, 1991; Blomberg et al, 1993). However, this method is time consuming and non-specific and therefore, other proteins usually co-elute with the receptors. Willemsen, (1993) attempted to isolate K88 receptors from porcine mucus by mixing it with pure K88 fimbriae, nevertheless, it was not possible to dissociate them, therefore, this method was only valid for their identification rather than isolation. Preparative gel electrophoresis was also used to isolate two brush borders receptors by Erickson et al, (1994). Recently Fang et al, (1999) have developed an affinity chromatography method for the isolation of K88 and K99 receptors. Using this method they identified 41 and 26 kDa receptors for *E. coli* K88ac in porcine intestinal mucus.

Anti-idiotypic antibodies bearing an internal image of a particular antigen have been widely used for identifying receptors that are recognized by the same antigen. They have been used to identify receptors for retinol binding protein and insulin (Sege and Peterson, 1978), chemotaxis (Marsaco and Becker, 1982) mammalian reovirus (Noseworthy et al, 1983), hormones and

neurotransmitters (Courand and Strosberg, 1991), and many other receptors. In this study fimbriae-Sepharose 4B affinity chromatography has also been used to isolate putative K88 receptors. Anti-idiotypic antibodies bearing an internal image to K88 fimbrial protein were then used to identify K88 receptors in the column eluate by ELISA and immunoblotting.

All the preparations of purified Ab2 recognized the receptors by ELISA. It appeared that the Ab2 preparations that were produced against pAb1 more strongly recognized the receptors as they yielded higher absorbance readings per unit of antibody protein than the corresponding Ab2 preparations produced against mAb1. This could be related to a difference in the affinity between both preparations or specificity or a difference in their titer. Monoclonal antibodies are unispecific to an epitope present on the K88 fimbriae, while the polyclonal antibodies recognize multiple epitopes on the K88 fimbriae due to their multispecificity. It is well known that K88 fimbriae are composed of major and minor subunits with the major being the part that attaches to the receptors (Klemm, 1985; Bakker, 1991; Smyth, 1994). It appeared that a mAb recognized only a part of the major subunit (10-12 amino acids), therefore, when injected into chicken they would have induced the production of anti-idiotypic antibodies with only a small fraction that bear an internal image of the K88 fimbriae. In contrast, the polyclonal antibodies would have contained a battery of antibodies that recognized every part of the fimbrial major subunit (the 264 amino acids compose the entire major subunit). Therefore, when pAb1 were injected into chicken, they would have produced Ab2 with a higher proportion that bear an internal image of the K88 fimbriae than Ab2 against mAb1. Anti-idiotypic antibodies produced against monoclonal IgM also appeared to be less effective than those produced against monoclonal IgG2b.

Receptor preparations were subjected to SDS-PAGE electrophoresis then transferred onto

nitrocellulose membranes and probed with either pure K88 fimbriae or the different Ab2 preparations. As expected, several proteins were detected by the K88 fimbriae. However, only one protein corresponding to 70 kDa was detected by Ab2 produced against pAb1, while no proteins were detected by Ab2 produced against either IgM or IgG2b monoclonal antibodies.

The reasons for the ability of fimbriae to recognize several receptors in contrast to the inability of Ab2 produced against pAb1 to recognize only one receptor may be attributed to five factors: i) although Ab2 mimic the K88 fimbrial antigen, they may have had different affinity for the receptors than the original K88 antigen, ii) the amount of specific Ab2 that mimic the K88 fimbriae may not have been sufficient to probe all the receptors, iii) the 70 kDa protein may be a dominant receptor with relatively higher affinity for the K88 fimbriae, and therefore for Ab2, iv) the SDS and mercaptoethanol treatments could have altered the conformation of the receptors in such a way as to completely or partially destroy its binding to Ab2 with exception of the 70 kDa which resisted denaturation (Metcalf et al, 1991) and v) the other proteins displaying MW 50-66 kDa might have bound non-specifically to the fimbrial column therefore, was not recognized by the anti-idiotypic antibodies. It is noteworthy that Ab2 produced against anti-K88 mAbs did not recognize any K88 binding protein. This could be due to the very low amount of Ab2 that had an internal image of the K88 fimbriae or might be due to its lower affinity for these proteins compared to K88 fimbriae or to Ab2 produced against pAb1.

When the purified receptors were stained for their sugar contents, the identified 70 kDa receptor did not seem to contain any sugar or it might have contained insufficient amount to have been detected. Interestingly, only one of the isolated proteins corresponding to 45 kDa appeared to have a sugar moiety which indicates that it is a glycoprotein. Metcalf et al, (1991) and Fang et al,

(1999) have identified a 40-42 kDa receptor in porcine intestinal mucus that appeared to be a glycoprotein. Willimsen, (1993) also identified three glycoprotein receptors in porcine intestinal mucus corresponding to 25, 35 and 60 kDa that bound K88 fimbriae and were of glycoprotein in nature. In our study we have identified four major proteins (40, 45, 50, and 70 kDa) with only one (45 kDa) appearing to be a glycoprotein. This protein could be the same protein reported by Metcalf et al, (1991) and Fang et al, (1999) as both are close in molecular weight and were glycoproteins as indicated by Metcalf et al, (1991), while the other three identified proteins may be identified for the first time since they differ from those reported in the literature in both molecular weight and identity. In conclusion, anti-idiotypic antibodies were used to identify a K88 receptor that was purified using affinity chromatography. This receptor might be of particular importance since it was strongly recognized by these antibodies.

5. Manuscript III

Studies on the Stability of Chicken IgY in Different Sugars, Complex Carbohydrates and Food Materials

5.1 Abstract

The effect of non-reducing sugars (sucrose, lactose and trehalose), complex carbohydrates (cyclodextrin and dextran), infant formula or egg yolk on the stability of purified chicken IgY was evaluated under different conditions. Regardless of the protectant that was used, about 20% of IgY activity was lost during freeze-drying except when in the presence of infant formula where the loss of IgY activity was approximately 75%. The lowest loss of activity (10%) was observed when no protectant was used. Trehalose was the best protectant followed by cyclodextrin and infant formula when IgY was stored for 6 or 14 wk at different temperatures. Sucrose, lactose and dextran were not effective as protectants under these conditions. IgY activity was completely lost after pepsin treatment in the presence of sugars or complex carbohydrates while 34 and 40 % of its activity was recovered when treated in the presence of infant formula and egg yolk, respectively. IgY was fairly stable after trypsin treatment with the recovery of residual activity being between 75-100% depending on the protectant. Finally, the effect of heat treatment on the stability of aqueous solutions of IgY was evaluated. IgY was stable at 50, 60 or 70°C for 30 min while complete loss of IgY activity was observed at 80 and 90°C in the presence of all protectants except infant formula and egg yolk were about 5% residual activity was observed. These results demonstrated that the addition of different compounds to IgY during freeze-drying or storage at different temperatures or subjecting them to enzyme treatments provided varying and in most cases considerable protection against loss of biological activity.

5.2 Introduction

Gastroenteritis is one of the major problems encountered in infants and young children in underdeveloped countries (Facon et al, 1995). In addition, elderly and immunocompromised patients are at great risk of such infections. Enterotoxigenic *Escherichia coli, Salmonella, Campylobacter, Vibrio cholera* and rotaviruses are the major microorganisms incriminated in gastroenteritis infections (Facon et al, 1995). Active immunization cannot be launched by immunizing against the pathogen in all people. Newborn infants, immunocompromised patients and those who are under chemotherapy treatment cannot respond effectively to vaccines, and will not produce adequate antibodies to specific pathogens. It has been reported that the newborn is capable of producing primarily IgM with the level being only 10% of that for adult (Benjamini and Leskowitz, 1991). Therefore, passive immunization is an alternative method. In this treatment, specific antibodies that are capable of blocking the adherence of microorganisms to the intestine or neutralizing them are administered orally. There are several reports on the efficacy of chicken IgY as therapeutic agents against dental carries and gastroenteritis in humans (Yolken et al, 1988 and Otake et al, 1991), against diarrhea in pigs (Kuhlmann et al, 1988; Yokoyama et al, 1992; Marquardt et al, 1999) and even against viral gastritis in mice (Bartz, et al 1980) .

It is well known that laying hens produce IgG that is transferred in high amounts from serum to the egg follicle (Kuhlmann et al, 1988). An estimation of 100-200 mg of IgG can be obtained from one egg (Kuhlmann et al, 1988), hence, chicken eggs can be used as a convenient source of antibodies.

Antibodies are proteins and are exposed to digestion and denaturation when passing through the stomach. Several studies have been conducted to evaluate the stability of these antibodies.

Shimizu et al, (1988, 1992, 1993 b) and Otani et al, (1991) studied the stability of the IgY under heat, acid as well as protease digestion. Chicken IgY was fairly stable under these conditions, however, its stability was studied in PBS or phosphate buffer without the addition of any protectants. Later, Shimizu et al, (1994) studied the stability of IgY in the presence of high concentrations of sugar. A sucrose concentration of 30-50 % was found to be highly effective in protecting the IgY from heat or acid denaturation and against high pressure as well. In all the previous studies, chicken IgY was extracted from the eggs and tested for its stability, however, the extraction process may involve organic solvents such as chloroform (Clarke et al, 1993) or propanol (Bade and Stegemann, 1984) which limits their value as food additives. Also this procedure may be unnecessary when IgY is incorporated in the feed as purification can result in reduced recoveries and additional expenses. Nevertheless, no studies have evaluated the effects of heat, acid and protease digestion on the IgY in the presence of a food matrix such as infant formula or egg yolk. Therefore, the objective of this study was to evaluate the stability of chicken IgY in the presence of infant formula and egg-yolk for the purpose of food fortification, and other sugars and lyoprotectants for the purpose of immunodiaganostics.

5.3 Materials and Methods

5.3.1 Materials

Trehalose, lactose, dextran, 2-hydroxypropyl-β-cyclodextrin (cyclodextrin for short), Complete and Incomplete Freunds adjuvants, p-nitrophenyl phosphate (ρNPP) were from Sigma (St Louis, MO). Sucrose was from Schwarz/Mann Biotech division of ICN Biomedicals Inc. (Cleveland, OH), rabbit anti-chicken alkaline phosphatase conjugate was from Jackson

Immunoresearch (Mississauga, ON), Falcon microtiter plates were from Fisher Scientific (Nepean, ON), infant formula (Enfalac) was from a local pharmacy, and the bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL). Freeze-dried egg-yolk powder was produced in the Department of Animal Science, University of Manitoba. All other chemicals were of analytical grade.

5.3.2 Chicken antibody production

White Leghorn hens that were 27 wk old were immunized with 500 μg affinity purified rabbit IgG2a in 0.5 ml of 0.01M phosphate buffered saline containing 0.14 M NaCl (PBS, pH 7.2) emulsified with 0.5 ml Freund's Complete Adjuvant. Booster shots of 250 μg/bird emulsified with Incomplete Freund's Adjuvant were given at monthly intervals. Eggs were collected after four boosts.

5.3.3 Antibody purification

Antibodies were extracted and purified from the egg-yolk using chloroform as an extraction agent following the method of Clarke et al, (1993).The semi-pure IgY fraction was subjected to high resolution anion exchange chromatography with a Pharmacia MonoQ HR 5/5 column (Pharmacia, Upsala, Sweden) using 50 % step gradient containing buffer A (0.05M phosphate buffer [pH 6.5]) and buffer B (0.3 M phosphate buffer [pH 6.5]). The volume of sample applied was 2 ml (10 mg/ml) and the protein was eluted at a flow rate of 1 ml/min at room temperature. The activity of IgY in the different fractions were evaluated by ELISA and the amount of protein was quantitated using the Pierce (BCA) Protein Assay. SDS-PAGE electrophoresis revealed the presence of only two bands

corresponding to the heavy and light chains of chicken IgY.

5.3.4 Enzyme treatment

Chicken IgY (1 mg/ml) digestion was performed with gentle shaking at 37°C. Freeze-dried IgY in the presence of different protectants and food matrices were reconstituted in 0.2 M acetate buffer (pH 2.5) for pepsin, or 0.2 M Tris-HCl buffer (pH 7.5) for trypsin. Enzymes were added to give a final ratio of 1:20 enzyme to substrate (w/w). Pepsin digestion was carried out at 37°C for 2 h while trypsin digestion was carried out for 4 h at the same temperature. The reaction was stopped by the addition of 2 M Tris-base or phenyl methyl sulfonyl fluoride dissolved in isopropanol for pepsin and trypsin, respectively.

5.3.5 Freeze-drying of IgY

Pure IgY was diluted to 1 mg/ml in solutions of 30% sucrose, lactose or trehalose; 20% infant formula; 5% cyclodextrin or dextran; or egg-yolk or water. Samples were rapidly frozen and then placed in a VirTis freeze-dryer model Genesis 25 LE (Gardiner, NY) for 24 h. Samples were removed and stored at -20°C until used.

5.3.6 Heat treatment

Antibody solutions (1 mg/ml) were heated at 50, 60, 70, 80 or 90°C in a water bath for 30 min in the presence of 30% sucrose, lactose or trehalose; 5% cyclodextrin or dextran; 20% infant formula; egg-yolk or water as a control.

5.3.7 Enzyme linked immunosorbent assay (ELISA)

The residual activity of IgY after each treatment was evaluated by ELISA. Briefly, 96 well microtiter plates were coated with 1 μg/well of rabbit IgG2a in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. Subsequently, plates were washed six times with 0.01 M PBS (pH 7.2) containing 0.05% Tween 20 (PBST), filled with 200 μl/well of 5% skim milk in PBST and incubated for 2 h at 37°C. Chicken anti-rabbit IgG2a (0.4 μg/well) in PBST was added to the plates, and incubated at 37°C for 1.5 h. After a washing step, 100 μl of alkaline phosphatase conjugated rabbit anti-chicken IgY diluted to 1:5000 in PBST was added to each well and incubated for 1h at 37°C. Finally, the color was developed by the addition of 100 μl/well of alkaline phosphatase substrate (pNPP), and plates were read at 405 nm using a microtiter plate reader (Model 450, BioRad Laboratories). All results were the average of triplicate analyses.

5.4 Results and Discussion

The use of chicken antibodies as therapeutic agents has several advantages over mammalian antibodies. The ease and continuity of their production makes them the antibodies of choice. In addition, their presence in the egg yolk offers an important advantage as they can be obtained without bleeding (Otani et al, 1991). However, using antibodies as diagnostic, therapeutic or food ingredients exposes them to conditions that might affect their efficacy. Antibodies might loose some or all of their activity when incorporated with food in the process of fortification. Furthermore, the gastrointestinal acidity and the digestive enzymes might have a detrimental effect on them while passing through the gastrointestinal tract (Shimizu et al, 1993a). Therefore, it is of prime importance to evaluate the stability of chicken IgY under conditions similar to those in the stomach in terms of

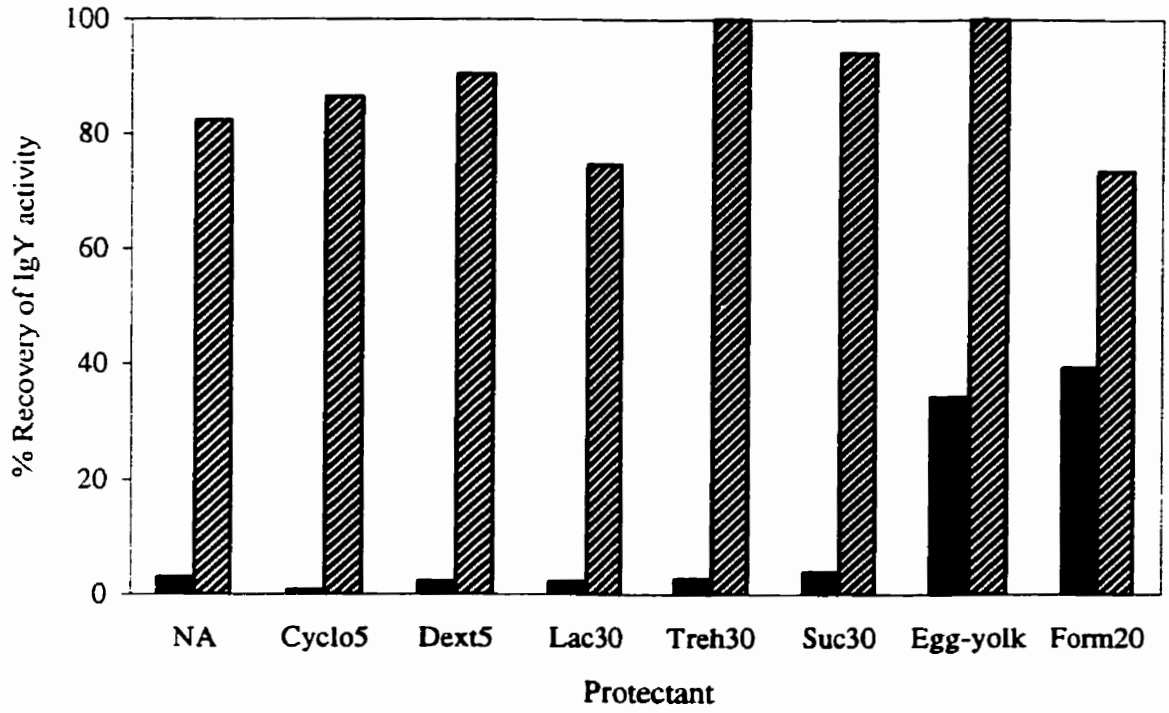
the combined effect of pepsin and acidity in addition to the effects of freeze-drying, storage and heat treatment.

5.4.1 Combined effect of proteases and acidity on the stability of chicken IgY

Chicken antibodies were incubated in both pepsin (pH 2.5) and trypsin (pH 7.5) at a ratio of 1:20 enzyme to substrate for 2 and 4 h, respectively. Figure 5.1 shows the percent of original activity after both treatments. The activity of IgY after pepsin treatment was almost completely lost in the presence of the three sugars, dextran or cyclodextrin as well as when no protectant was used. In contrast, the recovery of IgY when incubated with pepsin in the presence of infant formula or egg yolk was 39 and 34 %, respectively.

Pepsin digestion of mammalian IgG at a low pH usually leads to the production of $F(ab')_2$ fragments which continues to be reactive (Shimizu et al, 1988). However, this was not the case when chicken IgY was digested at a low pH, as the antibody lost most of its activity which indicates that digestion did not result in the production of active $F(ab')_2$ or Fab fragments as reported with IgG (Shimizu et al 1988; Otani et al, 1991; Shimizu et al, 1992 and Shimizu et al, 1993b). In this respect, it appears that chicken IgY resembles mouse IgG2b which is highly susceptible to pepsin digestion without the production of any active fragments. However, the particular susceptibility of mouse IgG2b to proteolysis was related to the larger hinge region (Parham, 1983), while the susceptibility of chicken IgY may be due to the high flexibility imparted by the lack of hinge region (Pilz et al, 1977). Further, Dreesman and Benedict, (1965) related the excessive susceptibility of IgY to proteolytic digestion to the weakness of the intramolecular non-covalent bonds between its heavy and light chains. In addition, the lower contents of β -sheets in IgY may possibly contribute to the low stability of IgY conformation (Shimizu et al, 1992).

Figure 5.1: Recoveries of chicken IgY activity when treated with pepsin [1:20 enzyme/substrate (E/S) ratio ■]; trypsin (1:20 E/S ▨) in the presence of PBS (NA); 5% cyclodextrin (Cyclo-5) or dextran (Dext-5) ; 30% lactose (Lac-30) , trehalose (Treh-30) or sucrose (Suc-30); egg yolk or 20% infant formula (Form-20). Values are average of triplicate analysis.


100
80
60
40
20
0
% Recovery of IgY activity
NA
Cyclo5
Dext5
Lac30
Treh30
Suc30
Egg-yolk
Form20
Protectant

The ability of egg-yolk and infant formula to stabilize IgY in contrast to the results obtained with the sugars and dextran may be related to their considerable buffering capacity or protective effects. The two food products could have elevated the pH of the solution which could have decreased pepsin activity, hence the IgY would not have completely digested. A similar situation may also occur *in vivo*. Pepsin may also targeted proteins other than IgY in the two products thereby sparing IgY from digestion. The choice of these two food products was not arbitrary. It is proposed that infant formula be fortified with specific IgY to protect infants against gastrointestinal pathogens. On the other hand, egg yolk is edible and therefore, can be used directly in certain foods without the need for the antibody purification.

In contrast to the pepsin digestion, trypsin treatment did not seem to have a detrimental effect on IgY. Complete recovery (100%) of the original IgY activity was observed when it was incubated in a trypsin solution in the presence of trehalose or egg yolk. Residual activities of 94, 90, 86, 82, 75 and 74 % were observed for sucrose, dextran, cyclodextrin, buffer, lactose and infant formula, respectively. It appears that IgY is more resistant to the effects of trypsin compared to pepsin . It is also apparent that infant formula or lactose did not confer any additional protection against tryptic digestion compared to the control. The high stability of IgY in the presence of trypsin indicates that once it passes the acidity of the stomach, it retains most of its activity and therefore, can combat or minimize the effect of intestinal pathogens. Furthermore, the dwell time of food in the stomach is estimated to be between 2-3 h for solid food and a shorter period for liquid food (Wardlaw and Insel, 1993). In infants this time is even shorter and the pH of their stomach contents is higher (4-4.5), thus, the damage to ingested IgY due to pepsin will be reduced (Shimizu, et al, 1988). The ability of IgY to survive digestion in the gastrointestinal tract is further supported by the

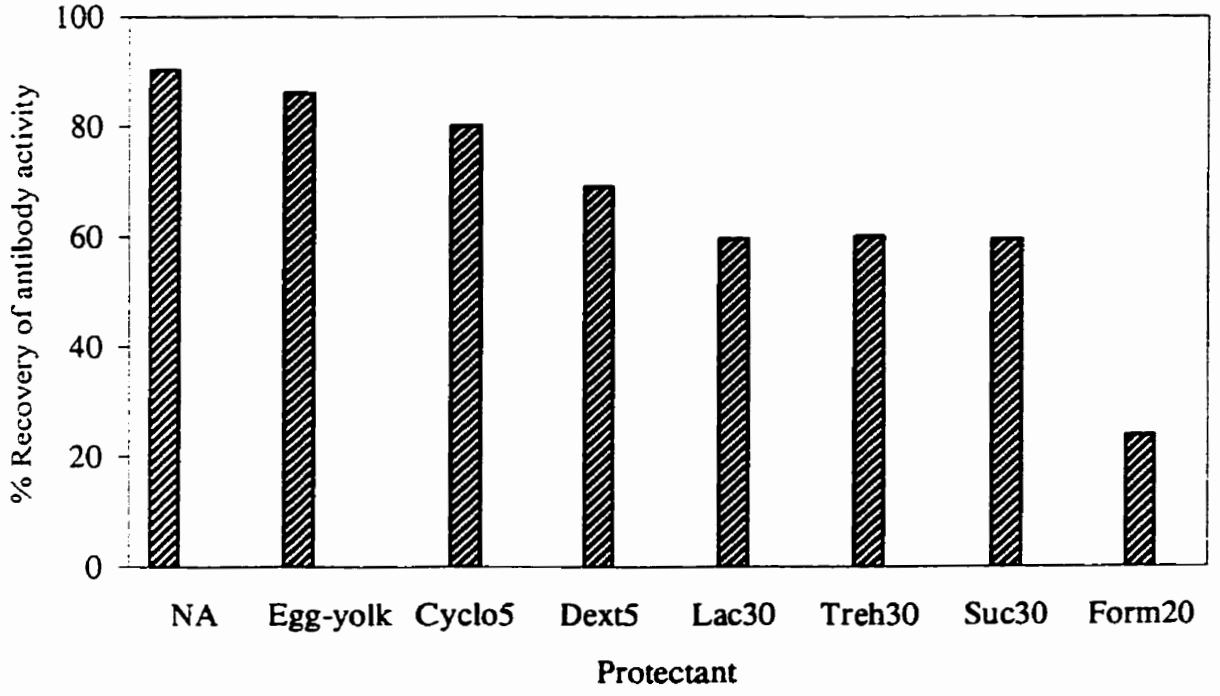
observation that egg-yolk antibodies can provide complete passive protection against *E. coli* infection in pigs (Yokoyama et al, 1992; Marquardt et al, 1999).

5.4.2 Effect of freeze-drying and storage temperature on the stability of chicken IgY

Freeze-drying and reconstitution of antibodies and biological materials can have a detrimental effect on protein structure and function (Ressing et al 1992). In the present study the effect of freeze-drying on the stability of IgY in the presence of non-reducing sugars, complex carbohydrates, egg yolk and infant formula was studied. The results revealed that the best recovery of IgY activity was observed when no cryoprotectant was added (90 %) or when egg-yolk was added (86%, Figure 5.2). The recovery of activity was slightly lower with the dextran and considerably lower with the sugars. The infant formula offered the least recovery as only 24% of the original activity of the antibody was recovered (Figure 5.2) regardless of concentration (data not shown). Ressing et al, (1992) reported a slightly higher recovery of mouse IgG2a (90%) when freeze-dried regardless of the cryoprotectant that was used. In our study a lower recovery was observed with IgY which could be related to the higher susceptibility of the IgY to denaturing conditions than mammalian immunoglobulins. Ressing et al, (1992) also reported that cyclodextrin was the best cryoprotectant which may be related to its ability to accommodate IgG in its cyclic cavity. Pikal et al, (1990 a , 1990 b) proposed that the cryoprotective effect of cyclodextrin may be related to its higher collapse temperature and its ability to remain amorphous during freeze-drying, while sugars and dextran are less amorphous and have lower collapse temperatures. However, these factors are not important in the current study as cyclodextrin did not enhance the stability of IgY.

It is known that the presence of sugars stabilize certain enzymes during freeze-drying due to

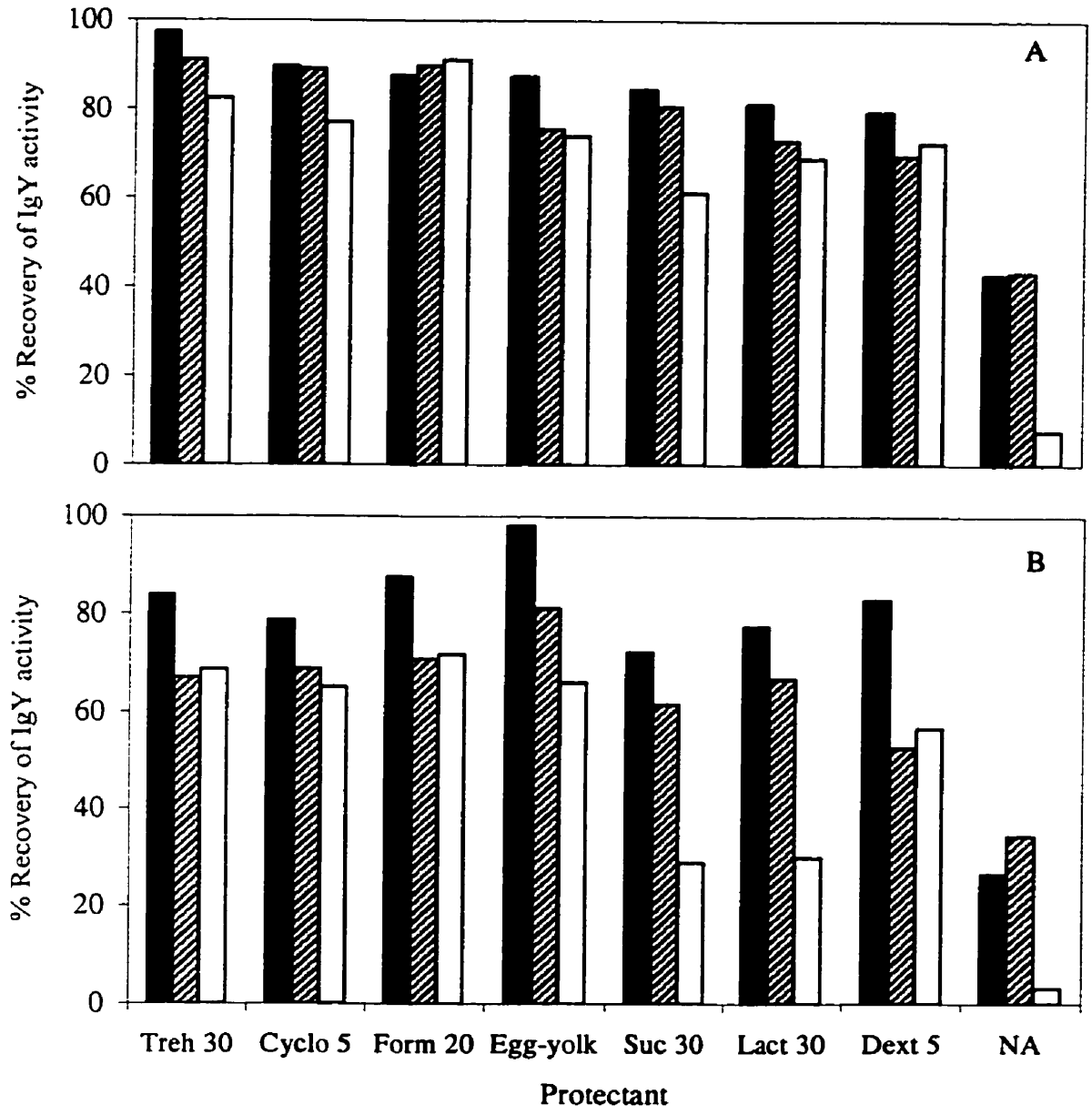
Figure 5.2: Recoveries of chicken IgY activity when freeze- dried in PBS (NA) ; egg yolk; 5% cyclodextrin or dextran.; 30% lactose, trehalose or sucrose; or 20% infant formula. Values are average of triplicate analysis.


100
80
60
40
20
0
% Recovery of antibody activity
NA
Egg-yolk
Cyclo5
Dext5
Lac30
Treh30
Suc30
Form20
Protectant

preferential hydration of the proteins (Carpenter et al ,1987b). However, it appears that the effect of any sugar as a cryoprotectant on protein stability depends on the nature of the protein and sugar. For instance, trehalose did not provide any exceptional properties in protecting IgY during freeze-drying while it was highly effective in protecting phosphofructokinase (Carpenter et al, 1987a) and bacterial cells (Leslie et al, 1995). The very low recovery of the IgY in the presence of the infant of formula is noteworthy. It is composed of different constituents including fat, proteins, carbohydrates, and several minerals and vitamins. Collectively these may have produced a damaging effect to the antibody during the freeze-drying process.

Freeze-dried IgY was stored for 6 wk at 21, 37, and 50°C and the residual activity was determined by ELISA (Figures 5.3a and b). Trehalose, cyclodextrin and infant formula were found to be the best potectants with over 80% of the original IgY activity recovered at all temperatures. Egg-yolk, sucrose, lactose and dextran provided slightly lower protection. However, IgY lost almost half of its activity at 21 and 37°C and all activity at 50°C in the absence of any cryoprotecants. Trehalose has been reported to be the cryoprotectant of choice during freeze-drying of bacteria (Leslie et al, 1995), mouse IgM (Drabar et al, 1995) and phosphofructokinase (Carpenter et al, 1987a). This could be due to the non-reducing nature of trehalose compared to sucrose which is a reducing sugar especially in the presence of the active amino groups of proteins. Therefore, prolonged storage of antibodies at ambient or higher temperatures in the presence of sucrose leads to the browning reaction, which damages proteins (Draber et al, 1995). In this experiment, extensive browning reaction was observed in IgY when stored in the presence of sucrose and lactose. Slight browning was also observed in the presence of infant formula, while no browning or change in color was observed in IgY stored in the other cryoprotectans. These observations could explain the high

Figure 5. 3a and b: Recoveries of chicken IgY activity when freeze-dried in the presence of 30 % trehalose, sucrose or lactose ; 5 % cyclodextrin or dextran; 20% infant formula; egg yolk or no additives (NA) and when stored for 6 (a) and 14 (b) wk at 21 ■, 37 ▨ and 50°C □. Values are average of triplicate analysis.


100
80
60
40
20
0
% Recovery of IgY activity
A
100
80
60
40
20
0
% Recovery of IgY activity
B
Treh 30
Cyclo 5
Form 20
Egg-yolk
Suc 30
Lact 30
Dext 5
NA
Protectant

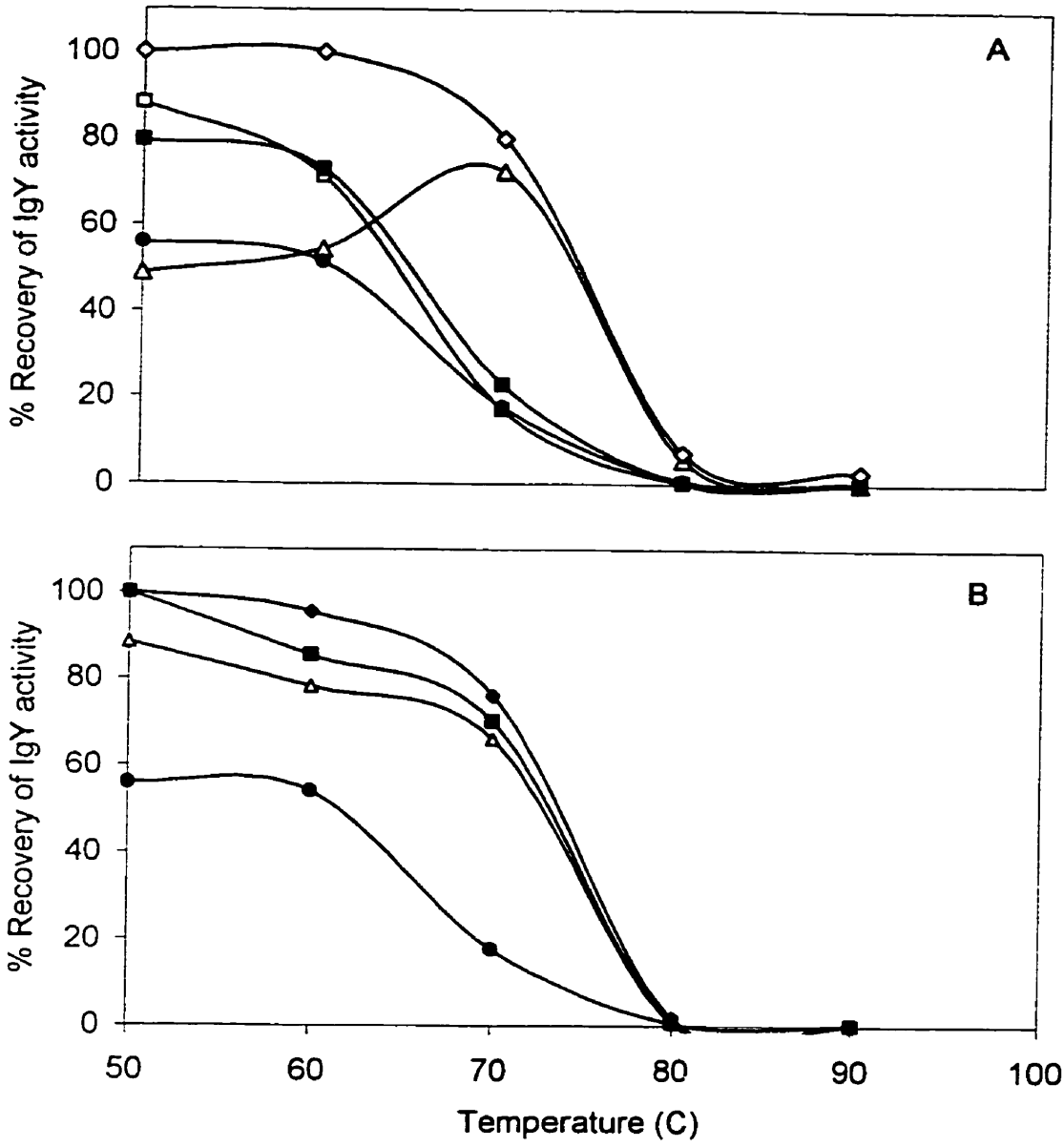
recovery of IgY in the presence of trehalose and cyclodextrin compared to sucrose and lactose. The infant formula and egg yolk appeared to confer similar protection to that of trehalose and cyclodextrin. Unlike sugars, when reconstituted prior to testing, large aggregates were noticed. It appears that the formation of such aggregates shielded the antibodies from the temperature effect during storage, thus, high recovery was observed. It has been reported that bacterial cells or their spores were able to resist killing to a greater degree when exposed to heat in a medium containing fat, carbohydrate or protein than when exposed to heat while suspended in a buffer (Splittstoesser et al, 1986). Therefore, the presence of these nutrients in both infant formula and egg yolk provided partial protection of IgY when stored at elevated temperatures.

IgY also continued to be fairly stable without much additional loss in activity when the storage period was extended to 14 wk except when IgY was stored at the higher temperature (50°C) in the presence of sucrose and lactose. The recovery of IgY activity in the presence of these sugars was only 30% of the original activity and the preparation appeared to be much darker due to extensive browning reaction. The freeze-drying studies therefore suggest that no cryoprotectant is required during freeze-drying of these samples. However, the storage for long periods of time at higher temperatures can result in considerable loss of IgY activity if there are no cryoprotectants or if the concentration of reducing sugars is high. Agents such as trehalose, dextrans, egg yolk or infant formula offer considerable protection against thermal inactivation of IgY during storage. Infant formula, however, should be added after freeze-drying so as to avoid loss of activity during freeze-drying.

5.4.3 Effect of heat treatment in an aqueous medium on the stability of chicken IgY

The heat stability of chicken IgY was evaluated by ELISA after incubating the antibody for 30 min at 50, 60, 70, 80 or 90°C in an aqueous solution in the presence of sugars, egg yolk, infant formula and complex carbohydrates. Very little loss of IgY activity was observed with any of the treatments when the antibody was exposed to 50°C except for the control (no additive) and infant formula (Figure 5. 4 a and b). However, at 60°C the highest recovery of IgY activity was observed in the presence of egg yolk and sucrose with the recovery being 100 and 96 %, respectively. A moderate loss of IgY activity at 60°C was observed in the presence of all sugars and other protectants except infant formula and the control where half of IgY activity was lost. IgY retained about 70% of its activity when incubated at 70°C in the presence of sugars, infant formula and egg yolk, while only about 20% of its activity was recovered when heated in the presence of cyclodextrin and dextran. The later two protectants compared to the control did not offer any additional protection to IgY at higher temperatures, and therefore are considered to be ineffective as thermal protectants. It is noteworthy that the recovery of IgY in the presence of infant formula increased with increasing temperature. Aggregation of the infant formula at the higher temperatures may have provided a physical shield for the IgY antibodies. IgY activity was completely lost at 80°C however, 5 and 7 % activity was recovered when heated in the presence of infant formula and egg yolk, respectively. From the above results it appeared that the IgY is stable in the presence of sugars, egg yolk and infant formula even at 75°C. These results are in agreement with these obtained by Shimizu et al, (1988, 1994) where they also reported that the heat stability of IgY is comparable to that of mammalian IgG. These studies therefore, demonstrated that IgY is relatively stable to high temperatures (70°C) in the presence of its natural matrix (egg-yolk) and the sugars including

Figure 5. 4a and b: Recoveries of chicken IgY activity when heated at 50, 60, 70, 80 or 90°C for 30 min in the presence of a) 5% cyclodextrin, (◇) or dextran (■), 20% formula (△), egg yolk(◇) or no additives(•) ; or b) 30% sucrose (♦), lactose (■), trehalose (△) or no additives(•). Values are average of triplicate analysis.


A
B
% Recovery of IgY activity
% Recovery of IgY activity
100
80
60
40
20
0
50
60
70
80
90
100
Temperature (C)

reducing sugars. Presumably these sugars did not interact with the amino groups of the IgY under these conditions.

In conclusion, the results from the present study indicated that certain sugars, especially the non-reducing sugars, infant formula and egg yolk are good thermoprotectants of IgY while cyclodextrin and dextran were less effective. Further, egg yolk and infant formula but not the other compounds protected IgY against the combined effects of pepsin and low pH. None of the cryoprotectants provided additional protective effects on IgY during freeze-drying, except for infant formula which was detrimental to IgY stability. Therefore, if infant formula is to be fortified with IgY against specific pathogens, it is recommended that it be added to the formula after freeze-drying.

6. General Discussion

Enterotoxigenic *E. coli* strains bearing K88 fimbriae cause diarrhea in neonatal and post-weaned piglets by colonization of the intestine and subsequent secretion of enterotoxins (Conway et al, 1990; Willemsen, 1993). Neonatal piglets are extremely sensitive to *E. coli* K88 infection. Normally, they are passively protected during the first few days by immunoglobulins found in the mothers colostrum. However, this source of antibodies dramatically declines shortly after parturition leaving the neonates prone to infection by *E. coli* K88.

Antibiotics have been the therapy of choice for these infections but the appearance of multiple drug resistance has put limitations on their use (Hays et al, 1986). An attractive alternative approach is the use of therapeutic antibodies that confer passive immunity. One source of immunoglobulins is the blood obtained from slaughtered pigs. Elliot, (1978) reported that the addition of spray-dried plasma, obtained from porcine blood to piglets feed maintained a 66 % survival rate compared to none for control groups. Spier et al, (1989) also reported that horses suffering from endotoxemia were protected by serum from horses that were immunized against the toxin. Monoclonal antibodies were also used for treatment of some diseases in domestic animals. Sherman et al, (1983) reported a 29 % mortality in calves treated with monoclonal antibodies against K99 compared to 82% mortality in the control group. The high costs associated with their production rendered this approach impractical.

Chicken IgY, however, is a cheap source of specific antibodies that can be used to combat several intestinal disorders. Over 200-300 eggs/year can be obtained from one bird immunized with a certain antigen with the yield being about 150 mg of pure IgY per egg (Marquardt et al, 1997). In addition the IgY need not to be purified, instead, it can be administered in its freeze-dried or spray

-dried form to animals through the feed or by gastric intubation (Marquardt, 1999). Complete passive protection of neonatal piglets against fatal enteric colibacillosis was reported by Yokoyama et al, (1992) when piglets were treated with chicken IgY that were produced against fimbrial adhesions of *E. coli* K88, K99 and 987P.

The problems and hazards that usually accompany the use of conventional vaccines has led to the development of new vaccine alternatives. Manuscript I details the production of anti-idiotypic antibodies that bear an internal image to the K88 fimbrial antigen. In this study anti-idiotypic antibodies (Ab2) were produced against both anti-K88 monoclonal (mAb1) and polyclonal (pAbl) antibodies. The ability of Ab2 antibodies to inhibit the binding of pure K88 fimbriae to Abl was evaluated by competitive ELISA. The results demonstrated that the Ab2 produced against pAb1 were twice as effective as Ab2 produced against mAb1. This could be related to differences in the specificity and affinity between pAb 1 and mAb1 for the K88 fimbrial antigen.

Despite this difference, Ab2 prepared against both pAbl and mAbl were able to confer complete protection (100 %) in mice when it was used as a vaccine several weeks prior to an *E. coli* K88 challenge. However, when the antibodies were administered orally to piglets before and after an *E. coli* K88 challenge, only partial protection (50% survival rate) was observed for the Ab2 treated groups compared to 12.5% survival rate for the control group. Furthermore, piglets that survived in the treated groups had milder diarrhea in the second and subsequent days than the non-treated piglets. These data suggest that anti-idiotypic antibodies can also provide partial protection against enteric diseases.

Anti-idiotypic antibodies are believed to compete with *E. coli* K88 for binding to the K88 intestinal receptors. Therefore, this property was used to identify K88 receptors in porcine intestinal

mucus. One receptor was identified by the Ab2 that was produced against pAbl, while the Ab2 that was produced against mAb1 failed to recognize any K88 binding protein. It appeared from the results that the idiotypic polyclonal antibodies as indicated by the competitive ELISA and the receptor identification studies were more effective than idiotype monoclonal antibodies in the production of beneficial anti-idiotypic antibodies. Furthermore, these studies also indicated that the Ab2 produced against $F(ab')_2$ fragments of rabbit IgG2a did not offer any additional efficiency over these obtained with the intact IgG2a.

IgY seems to be an excellent reagent for therapeutic, prophylactic and for receptor bindings studies. IgY stability is an important criteria that affects its performance, therefore, the effect of freeze-drying, heat treatment, pepsin and trypsin digestion on its stability was investigated in the presence of protectants. Among the different protectants, egg-yolk and infant formula appeared to be good protectants except for the infant formula which was detrimental to the IgY activity during freeze-drying. It should, therefore, be added to the preparation after freeze-drying. Overall, IgY appeared to be fairly stable under different conditions and indeed can be used for therapeutic and diagnostic purposes.

7. Conclusions and Recommendations

Based on these studies it can be concluded that:

1. The anti-idiotypic antibodies (Ab2) that were produced against polyclonal antibodies (Ab1) were superior to those produced against monoclonal antibodies (mAb1) in terms of the proportion of Ab2 that bear internal images to the K88 fimbrial antigen.
2. Among the pAb 1 used to produce Ab2 there were no difference in relative efficacy of Ab2 when it was produced against either the whole IgG molecule or its $F(ab')_2$ fragment. In contrast, among the mAb 1 used to produce Ab2 there was a difference in the relative efficacy of Ab2 as measured by competitive ELISA. In this comparison, Ab2 produced against IgM mAb1 were able to effectively compete for *E. coli* K88 fimbriae better than Ab2 produced against IgG2b mAbl.
3. Anti-idiotypic antibodies proved to be good immunizing agents as they produced complete (100 %) protective immunity when they were used as surrogate antigens to immunize mice prior to an *E. coli* K88 challenge. However, there was no difference between the degree of protection produced by the two different Ab3; the ones that were originally derived from either pAb1 or mAbl.
4. The polyclonal anti-idiotypic antibodies (Ab2) that were produced against anti-K88 rabbit IgG2a and its $F(ab')_2$ fraction (pAbl) conferred only partial protection in piglets challenged by *E. coli* K88 when antibodies were administered orally. It appeared that the proportion of Ab2 that bore an internal image to *E. coli* K88 fimbrial antigen was not enough to block all the receptors and therefore prevent infection. Nevertheless, it was partially effective.
5. The anti-idiotypic antibodies were used to identify *E. coli* K88 putative receptors, as a result, a

70 kDa protein was identified in porcine intestinal mucus. However, the Ab2 produced against mAb1 failed to recognize any receptor which could be due to the low proportion of Ab2 that bore an internal image to the K88 firmbrial antigen.

6. Non-reducing sugars, infant formula, and egg-yolk are good thermoprtoectants of IgY, while cyclodextrin and dextran were less effective.
7. Only egg-yolk and infant formula provided partial protection of the IgY against the combined effects of pepsin and low pH.
8. None of the protectants used in the study provided any additional protection to IgY during freeze-drying of IgY, except for infant formula which was detrimental.

Recommendations:

1. Further studies should be conducted to test the activity of IgY fed to piglets and obtained from their intestine after it passes through the stomach, so that its stability is evaluated under virtual conditions.
2. Polyclonal idiotype antibodies (pAb1) rather than monoclonal idiotype antibodies (mAb1) should be used for the production of polyclonal anti-idiotypic antibodies.
3. Further studies should be directed toward the production of only the antigen combining sites of the Ab1. These can be produced by the phage display technique. The combining site will not provoke the immune system by themselves, thus, can be conjugated to an inert protein to produce more specific Ab2 that bear an internal image to the antigen of interest.

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