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Development of a Subunit Vaccine Against Bovine Diarrhea Caused by K99 Enterotoxigenic Escherichia coli and Bovine Viral Diarrhea Virus

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DEVELOPMENT OF A SUBUNIT VACCINE AGAINST BOVINE DIARRHEA

CAUSED BY K99 ENTEROTOXIGENIC *ESCHERCHIA COLI* AND BOVINE VIRAL

DIARRHEA VIRUS

BY

Emad Hashish

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2013

DEVELOPMENT OF A SUBUNIT VACCINE AGAINST BOVINE DIARRHEA

CAUSED BY K99 ENTEROTOXIGENIC *ESCHERCHIA COLI* AND BOVINE VIRAL

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

AC: Adenylate cyclase

BDV: Border disease virus

B-PER: Bacterial protein extraction reagent

BVDV: Bovine viral diarrhea virus

CF: Colonization factors

CFA: Colonization factor antigen

CFTR: Cystic fibrosis transmembrane conductance regulator

CFU: Colony-forming unit

cGMP: Cyclic guanosine monophosphate

cp: cytopathic

CPE: cytopathic effect

CSF: classical swine fever

CT: cholera toxin

DCs: Dendritic cells

DMEM: Dulbecco's modified eagle medium

EAEC: Enteroaggregative *Escherichia coli*

ECD: Extracellular binding domain

E. coli: *Escherichia coli*

EHEC: Enterohaemorrhagic *Escherichia coli*

EIA: Enzyme immunoassay

EIEC: Enteroinvasive *Escherichia coli*

ELISA: Enzyme-linked immunosorbent assay

EPEC: Enteropathogenic *Escherichia coli*

ETEC: Enterotoxigenic *Escherichia coli*

Fan: fimbrial adhesin K ninety-nine

FBS: Fetal bovine serum

FCA: Freund's complete adjuvant

FIA: Freund's incomplete adjuvant

GC-C: Guanylate cyclase C

GI: Gastrointestinal

GM1: Monosialotetrahexosylganglioside

GTP: guanosine-tri-phosphate

HRP: Horseradish peroxidase

IBMX: Isobutylmethylxanthine

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IP: Intraperitoneal

IPEC: Porcine intestinal epithelial cell

IPTG: isopropylthio-β-galactoside

IRF3: Interferon regulatory factor 3

LB: Luria-Bertani

LT: Heat labile enterotoxin

MEM: Minimum essential medium

MD: Mucosal disease

MOI: Multiplicity of infection

Ncp: Non-cytopathic

Ni-NTA: Nickel-nitrilotriacetic acid

OD: Optical density

ORF: Open reading frame

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PI: Persistent infected animal

PKA: Protein kinase A

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SOE: Splicing overlapping extension

ST: Heat-stable enterotoxin

STa: Heat-stable enterotoxin type Ia

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ABSTRACT

DEVELOPMENT OF A SUBUNIT VACCINE AGAINST BOVINE DIARRHEA

CAUSED BY K99 ENTEROTOXIGENIC *ESCHERCHIA COLI* AND BOVINE VIRAL

DIARRHEA VIRUS

EMAD HASHISH

2013

Bovine diarrhea is economically one of the most important problems in cattle industry. Enterotoxigenic *Escherichia coli* (ETEC), particularly strains expressing K99 (F5) fimbriae and heat-stable type I (STa) enterotoxin is the predominant bacterial cause of diarrhea in calves; whereas bovine viral diarrhea virus (BVDV) causes diarrhea and other illness to cattle at all ages. A vaccine that prevents against both K99 fimbrial ETEC and BVDV could benefit greatly to cattle producers worldwide. We hypothesized that a novel multivalent vaccine composed of K99, STa and BVDV E2 antigens could induce immunity for broad protection against bovine diarrhea. In this study we applied FanC, the major fimbrial subunit of K99, as a backbone to have a STa toxoid and the most immunogenic E2 epitopes embedded to construct 'FanC-STa-E2' fusion antigen, and examined this 'FanC-STa-E2' fusion antigen for immunity against ETEC and BVDV in a murine model for vaccine potential assessment. Adult female BALB/C mouse intraperitoneal (i.p.) immunized with 200 μg purified antigen with equal volume of adjuvant. Developed systemic and mucosal immune responses to K99, STa, and BVDV E2. Moreover, those antibodies showed abilities to block the *E. coli* K99 bacterial

adhesion by K99-receptor positive INT-407 and IPEC-J1 cells, neutralized the STa

enterotoxin, and also neutralized the BVDV infection. This suggests that this fusion

antigen has the potential to be developed as a broadly protective vaccine against bovine

diarrhea.

Chapter One

Literature Review

Introduction

Diarrhea is not a specific disease but it is a symptom of many diseases, which might be the principal or secondary symptom. Diarrheal infection causes morbidity and mortality among animals, particularly young animals and results in significant economic losses [2]. Indeed, diarrhea is the most significant disease in neonatal dairy calves worldwide [3]. Calves within the first months of life are usually at a greatest risk of developing diarrhea, while the incidence of diarrhea decreases with increasing age [4, 5]. Neonatal calf diarrhea results in significant economic losses and increases production costs due to treatment, diagnosis, labor and the veterinary intervention [6]. Although infectious diarrheal diseases reflect interaction between pathogen and hosts, and also environmental and management factors, enteric pathogens play the major role in causing diarrhea. It is reported that enteric pathogens are found associated with around 97.6% of outbreaks and were detected in 95.0% of the fecal samples collected from dairy and beef calves with diarrhea in Australia [7].

As ETEC is the predominant cause of calf diarrhea and BVDV is the main viral cause of diarrhea in cattle at all ages, the major focus in this study is on these two pathogens. ETEC strains cause severe watery diarrhea in calves and other young animals such as piglets [8]. BVDV can cause diarrhea and other illness, such as the widespread lesions in the gastrointestinal tract with diarrhea and respiratory symptoms, there is a severe form of BVDV called BVDV-mucosal disease complex (BVDV-MD) [9].

Vaccination

Vaccines prevent and control diseases in humans and animals. The first recorded use of a vaccine by the ancient Greek king of Pontus, Mithridates VI. in 1st Century, BC.. He tried to protect himself from being poisoned through the repeated consumption of small amounts of poisonous substances in honey (theriaca) [10]. The first veterinary vaccine was Pasteur's preparation to immunize chickens against fowl cholera [11]. Vaccination is an important approach to prevent or prophylaxis of the disease, by providing good immunological responses before natural infection taken place, so that to reduce the cost of therapeutic approaches. Vaccination programs generally aimed to control infectious diseases in man and animals and also to reduce or to prevent the morbidity and mortality. Enteric diseases, however, remain endemic and largely have no effective vaccines available [12]. Vaccines are expected to induce specific antibodies directed against infections by specific pathogens to confer the protection [13].

Traditional vaccines were attenuated either by serial passage of an agent in tissue culture or inactivation of an agent by chemical or physical means. But these vaccines have disadvantage including reversal to virulence in case of live attenuated vaccine that result in the production of disease. Genetic engineered vaccines through molecular biology, are thought to have great promise [14]. Different types of vaccines: killed whole cell bacterins, inactivated virus, live attenuated bacteria and viruses, and subunit including synthetic peptides and toxoid vaccines, are currently in use to control viral and bacterial infection [10, 15]. Subunit vaccines have become preferred over the live vaccine, because presents an antigen (protein or peptides) to the immune system without introducing pathological particles. The advantage of the Subunit vaccines is the

increasing in the safety concerns, less antigenic competition and selection of the most immunogenic peptides or protein which directly target and stimulate the immune system. However subunit vaccines require preparation and purification in a large volume and administered in appropriate confirmation to elicit desired immune responses [14]. Other approaches have also been attempted, including production of transgenic plants that produce edible antigenic proteins to induce immunity against pathogens including diverse enteric and non enteric pathogens [16]. This transgenic plant system has been demonstrated to express and produce foreign proteins that elicit specific immune responses in the murine system [17].

Advances in molecular biology allow scientists to isolate virulence genes from pathogens and to express these genes in vectors and to vaccinate with this DNA [10, 14]. This recombinant genetic approach leads to a concept of multivalent vaccine. Multivalent vaccine concept was tried in developing multiepitope subunit vaccine to protect against extraintestinal pathogenic *Escherichia coli* [18]. A single multivalent vaccine can be used against different diseases. An example is the multivalent DNA-based vaccine against rabies and canine distemper in mice and dogs[19]. In addition, like any traditional vaccines, a multivalent vaccine can be co-administrated with other vaccines simultaneously to provide broad protection. Co-administration reduces the number of injections that resulted in lowers vaccination cost [20]. However, before applying any vaccination program in a disease control program, careful consideration should be taken regarding political, practical, scientific and economic factors. Otherwise the wrong decision may result in waste of resources, the spread of disease, trade restrictions and the loss of public confidence [21]. The vaccine must be cost-effective to combat and

eradicate infectious diseases, as developing countries are typically endemic to infectious diseases [22].

Escherichia coli

Escherichia coli are gram negative rod shaped bacilli that typically colonize the gastrointestinal (GI) tract of humans and animals as a normal flora of the GI system. *E. coli* are mostly non-pathogenic, harmless bacteria and can be beneficial to the host by participating in vitamin metabolism [23] or by blocking establishment of enteropathogens through competing for limited nutrients or bacterial binding receptors [24]. They usually remain normal inhabitant in the intestinal lumen without producing any diseases. Only very limited number of pathogenic *E. coli* strains cause clinical diseases such as urinary tract infection, diarrheal disease, and meningitis [25].

Diarrheagenic *E. coli* strains are a group of the pathogenic *E. coli* causing diarrheal diseases in both humans and animals. Theses *E. coli* strains deliver toxins to host cells to cause fluid hyper-secretion in host epithelial cells to cause diarrhea. Once colonized, diarrheagenic *E. coli* strains exhibit remarkable versatility to cause diarrhea. Based on their virulence mechanisms, epidemiology and serotypes, six distinct groups were recognized: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC, enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) [25, 26].

Enteropathogenic *E. coli* (EPEC) causes persistant watery diarrhea in children less than two years old in developing countries [27]. Enterohemorrhagic *E. coli* (EHEC) occurs in industrialized nations, where adults and children can suffer from either bloody or non-bloody diarrhea or hemolytic uremic syndrome with Shiga toxins (Stx) production

[25, 26]. Enteropathogenic and enterohemorrhagic *E. coli* (EPEC and EHEC) share

several characteristics such as, locus of enterocyte effacement[28], pathogenicity island

(PAI), encoding a type III secretion system [29], capability to form attaching and effacing

(AE) intestinal lesions, and intimate attachment to the host cell [27, 30].

Enteroaggregative *E. coli* (EAEC) is responsible for travelers' diarrhea in industrialized

and developing countries characterized by aggregative adherence (AA) to HEp-2 cells in

culture[31]. Enteroinvasive *E. coli* (EIEC) strains cause diarrheal disease and dysentery

in young children in developing countries [31, 32] and food-borne outbreaks of enteric

disease in adults in industrialized countries [33]. High fever, malaise, abdominal cramps,

and overt dysentery are symptoms produced from the cytotoxins and enterotoxins

involved in EIEC infection [34].

ETEC is the major bacterial cause of diarrhea [26]. ETEC bacteria commonly

cause colibacillosis in humans, pigs and calves, but less common in dogs and cats.

Colibacillosis refers to adherence of bacteria to the microvilli of small intestinal epithelial

cells but with no induction of any morphological lesions, and enterotoxic activity caused

by produced enterotoxins which act locally on enterocytes to cause diarrhea.

Colibacillosis among the neonatal pigs and calves is directly responsible for high

economic losses [8]. Human ETEC is responsible for a high mortality rate of infants in

the developing countries, also is considered as the most common cause of diarrhea among

travelers from industrialized countries to the tropical or subtropical areas of the world

[26, 35-37]. ETEC diarrhea starts with ingestion of contaminated food or water. Theses

bacteria adhere to the small intestinal epithelial cells of the newborn or very young

animals through the production of thin (3-7 nm) proteinaceous surface appendages called

fimbriae or pili. Fimbriae are morphologically, biologically and antigenically different among various ETEC strains. Fimbriae ETEC bacteria colonized at the gut that allows bacteria to secrete enterotoxins to the target cells. That stimulates the small intestine for increased water and electrolyte secretion but prohibits fluid absorption, and leads to diarrhea [8].

Two types of virulence factors are identified in ETEC infections, the adhesive fimbriae and secretion of enterotoxins. Fimbriae and enterotoxins are different among different ETEC strains. ETEC strains causing newborn calves diarrhea carry adhesins K99 (F5) and/or F41 and porcine-type STa (pSTa) enterotoxins [8, 38], that is different from ETEC strains associated with diarrhea in suckling piglets that express K88 (F4) adhesins (K88ab, K88ac and K88ad) [39], F18, K99 (F5) or 987P (F6) adhesins [8] and occasionally F41 [40], F165 [41] or F42 adhesins [42]. The most common ETEC causing diarrhoea in pigs express K88 and LT and p STa or STb [8]. This study focuses on the K99 fimbrial ETEC strains that cause diarrhea in calves.

E. coli Pathogenesis

Two major virulence factors are associated with ETEC infection and pathogenesis. They are adhesions or fimbrial mediate ETEC colonization at host small intestine and enterotoxins including, the heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST) [8, 25, 26, 43]. Host specificity, mainly determined by the presence of specific receptors, effects outcomes of ETEC infections [8]. Colonized ETEC bacteria are able to elaborate the enterotoxins to host cell and fluid secretion that leads to diarrhea [8, 25]. ETEC associated with animal diarrhea produce several important adhesions or different types of adhesions, such as the F4(K88), F5(K99), F6(987P), F41, F42, F165,

F17 and F18 fimbriae, as well as different types of enterotoxins including, heat-labile (LT) and heat-stable (ST) enterotoxins which can be further classified in to LTh-I, LTp-I, LTlla, LTIIb, hSTa, pSTa, STb [8].

A. Colonization factors

Colonization of the host small intestine is a necessary prerequisite for ETEC to cause diarrheal disease. So that establishment of bacterial adherence to enterocytes is an important step to initiate ETEC infection and diarrhea. Colonization occurs by adhesion to the host mucosal surface [44, 45]. As stated before there are several types of fimbriae that are host specific. High species specificity of ETEC fimbriae has been discovered [36]. This adhesion is mediated by specific adhesins or fimbriae found on the outer surfaces of ETEC [46, 47]. Different colonization factors have been reported in human and animals, with more than twenty colonization factor antigens (CFA) in human ETEC strains and several fimbriae in animal ETEC strains that are responsible for the adhesion [48, 49]. Fimbriae are filamentous appendages on bacteria that allow colonization of host epithelial surfaces, that brings ETEC in close proximity to host cells and elaborates enterotoxins to epithelial cells [50, 51]. ETEC fimbrial adhesins mediate the attachment to the brush border of epithelial cells small intestinal microvilli, establishing colonization and resisting removal without inducing morphological lesions [8, 25].

ETEC strains associated with diarrhea in calves and lambs express two fimbrial adhesins called K99 (F5) and F41 [52]. Small intestinal adhesion and colonization by ETEC is facilitated by the fimbria-receptor interaction, and the susceptibility of young animals to ETEC infections is determined by the presences of receptors specially recognized by ETEC adhesions [43]. The presence or absence of adhesin receptor genes

is responsible for the host specificity of ETEC infection regarding adhesion and colonisation which would be different in different animal species. K99 fimbriae are responsible for the attachment of the bacteria to the host epithelium cells and facilitate subsequent bacteria colonization [37]. The K99 fimbriae specific receptor is the small intestinal glycolipid ganglioside N-glycolyl-GM3 (NeuGc-GM3) that binds laterally at numerous positions to the fimbriae [53, 54]. On the other hand, the degree of expression of host receptor genes is influenced by age and reflects the resistance or the susceptibility to adhesion by ETEC. The glycolipid receptors for F5(K99) [54] has been reported to be declined with increase of age of the animal [55]. The K99 fimbrial antigen was characterized as a single repeating protein subunit [56]. The major subunit called fimbrillin forms fimbrial structure and recognizes host receptor ganglioside [57]. The FanC has been reported as the major k99 fimbrial subunit act as adhesin at numerous positions along fimbriae and result in binding between the fimbriae and receptor , so that the adhesive properties is associated with the fimbriae [53].

B. Enterotoxins

Enterotoxins are plasmid-regulated exotoxins or extracellular proteins or peptides which are able to exert their actions on the intestinal epithelium [8]. After bacterial colonization to the small intestine using specific fimbriae, the ETEC is able to efficiently transmit the enterotoxins, which disrupt fluid homeostasis in intestinal epithelial cells, and result in functional change which lead to secretory diarrhea [8, 25]. ETEC has two major classes of enterotoxins, the large-molecular-weight heat labile toxin (LT) and the small-molecular weight heat stable toxin (ST), which are different in structure, function

and immunogenicity. LT predominantly secreted by human and porcine ETEC strains, while ST usually produced by ETEC of human, porcine and bovine origin [8].

1- Heat labile toxin

The heat labile toxin (LT) is very closely related to cholera toxin (CT) that is expressed by *Vibrio cholera* in function and structure [50, 58]. LT is a hexameric protein composed of a ring-shaped homopentameric B subunits of 11.5 kDa which strongly bind to its specific intestinal receptor (ganglioside GM1), and a single A subunit of 28 kDa which is responsible for the enzymatic activity of the toxin, and is proteolitically cleaved to yield A1 and A2 peptides joined by a disulfide bond [25]. The pentamer B subunits bind LT at host cell surface, then LT enters into the cell through an endocytic process. Heat-labile enterotoxins can be divided into two known categories, the LTI and LTII. LTI are expressed by ETEC associated with diarrhea in humans and animals, while LT-II, primarily found in animal *E. coli* isolates, has not been associated with disease [8]. Where LTI has two subclasses the LTh-I (human) and LTp-I (porcine), where LTII has two antigenic variants (LTIIa and LTIIb) [8].

Adenylate cyclase (AC) is the cellular target of LT, located on the basolateral membrane of polarized intestinal epithelial cells [25]. After binding to ganglioside GM1 receptors at the host epithelial cells through the B subunits, LT holotoxin is endocytosed and translocated into host cells [59]. The A1 fragments translocate into the cell and activate the adenylate-cyclase system through ADP-ribosylation of the alpha subunit of GTP binding protein Gs with the activation of the catalytic unit of cAMP dependent protein kinase, thereafter, adenylate cyclase is permanently activated by the ADP-ribosylation of the GTP-binding protein, leading to increased production of intracellular

cyclic AMP [38]. cAMP phosphorylates the membrane proteins leading to transepithelial ion transport disorders and intracellular accumulation of cAMP which elicits secretion by crypt cells and decrease absorption by villus tip cells [8, 25]. As a result, hypersecretion of electrolytes and water into intestinal lumen occurs, and diarrheal disease is induced [60].

2- Heat stable enterotoxins

Heat stable enterotoxins (ST) are small monomeric proteins, classified into two classes, the STa and STb or STI and STII respectively. Two types of STa are recognized: the pST (ST porcine or STIa) and hST (ST human or STIb) [61]. STp and STh have a 50% sequence homology, and they are identical in the 13 residues necessary and sufficient for enterotoxicity, six of them are cysteines which form three intramolecular disulfide bonds [8, 25]. The STa major receptor is membrane spanning enzyme belongs to the receptor cyclases family called guanylate cyclase C (GC-C) which located on the apical membrane of intestinal epithelial cells, where the ligands binding to the extracellular domain stimulates the intracellular enzymatic activity [61]. The GC-C receptor which spanned the cell membrane and composed of the extracellular binding domain (ECD), single small transmembrane domain, single transmembrane helix, the cytoplasmic intracellular protein kinase homology domain (KHD), catalytic domains and a carboxy-terminal domain [62, 63].

STa is synthesized as a 72 amino acid precursor proteins consisting of a pre signal peptide, a pro sequence, and the carboxy-terminal enterotoxin [64, 65], and synthesized as an intracellular pre-pro-STa polypeptide [66]. The amino acids 1-19 is considered a signal peptide that directs the translocation of the pre- pro-STa polypeptide across the

inner membrane [65]. Where the outer membrane translocation is accompanied by the cleavage of the pre-pro-STa polypeptide into the mature peptides, where pSTa & hSTa have 18- or 19 mature amino-acid peptide respectively which are cleaved by a SecA-dependent export pathway, and has a molecular mass of ca. 2 kDa [66, 67]. The mature STa has three intramolecular disulfide bonds between six cysteines.

The STa binding is followed by the stimulation of GC activity and increased intracellular cGMP levels [68-70], with the activation of cGMP-dependent kinases [70]. Then phosphorylation of the CFTR chloride channel leads to secretion of Cl^- ions into the intestinal lumen [8, 62]. It has been reported that the activation of cGMP-dependent protein kinase II (cGK II or PKG) is sufficient and necessary to activate Cl^- secretion [71]. The cGK II knock out mouse has been reported as not responsive to ST, guanylin, or cGMP [72]. Finally, the net outcome is the stimulation of guanylate-cylase system with intracellular accumulation of cGMP which induces a reduced absorption of water and electrolytes on villus tips with extensive excretion of Cl^- and water into the crypt cells, causing dehydration and diarrhea causing the stimulation of chloride secretion and/or inhibition of sodium chloride absorption, resulting in net intestinal fluid secretion [8].

Since STa is a small molecule, it is not immunogenic in its natural form. Several approaches have been developed to enhance its immunogenicity as a vaccine target. One of the problems is that the actual amount of STa toxin produced by an ETEC strain during an ETEC infection in humans is unknown. Since STa is unable to produce anti-ST antibodies during natural ETEC infections, strategies must be developed to induce neutralizing anti-STa antibodies. It could be difficult to achieve complete ST

neutralization. Immunity may then depend on partial protection against the ST toxin to effectively reduce the ETEC disease burden and pathogenesis [67].

Escherichia coli **K99 fimbriae**

ETEC K99 fimbrial antigen is classified based on antigenic and genetic characteristic. Structurally, K99 is composed of fimbrillin that forms the main part of fimbriae and adhesin which recognizes carbohydrate receptors on the surface of host cells [54, 56]. Genetically, K99 ETEC fimbriae have eight different genes involved [73]. The genetic determinants for ETEC K99 fimbriae requires the in vivo biosynthesis of eight unique polypeptides genes named fanA, fanB, fanC, fanD, fanE, fanF, fanG, fanH [74]. Those genes reside on a large plasmid, encodes six structural genes clusters and eight subunits fanA-fanH [75]. Those subunits have been analyzed and the function has been determined. The full operon has been cloned [76, 77]. K99 is transmissible, being genetically encoded on an 87.8-kb plasmid [1]. Organization of this Fan operon is complex, and is regulated at the transcriptional level by a variety of factors [73, 78]. Several factors affect the expression of K99, it has been shown to be dependent upon cyclic AMP (cAMP) [79], cAMP receptor protein (CRP) [74, 80], and leucine-responsive protein (LRP) [81].

The K99 operon composed of three separately regulated gene clusters: region I, encoding fanA to fanD; region II, encoding fanE and fanF; and region III, encoding fanG and fanH [74]. The expression of region I is not dependent on region II or III, as the fanC expression was not altered when region II and III genes were removed [73]. Two open reading frames located upstream of FanC gene are named as FanA with a molecular mass of 11.000 daltons and FanB with a molecular mass of 10.500 daltons, and these two

genes served as a regulatory elements which control the expression of K99 fimbriae [82]. A region with dyad symmetry within FanA supposed to represent the *cis*-acting element and allow the FanA and FanB to interact with the termination factors preventing the termination at T_1 and T_2 [83].

FanC is the most highly expressed protein in the Fan operon [73], the major structural subunit and immunogenic polypeptide of K99 fimbriae [84]. It encodes 181 amino acid precursor protein [85], with a molecular weight of 18.500 daltons [85, 86], those amino acid residues composed of a single repeating protein [73, 86]. FanD served as a platform protein presumed to be an usher, and codes for a polypeptide of 783 amino acid residues with a molecular mass of 84549 daltons for the mature FanD protein, and showed a significant sequence homology with FaeD, the large outer membrane protein encoded by the K88 operon [87]. FanE starts with GTG (valine) as starting codon which overlapped by fanD stop codon and composed of 207 amino acids and 3 restriction enzyme sites HincH, CiaI and AvaI, while fanF with starting region overlapped by the terminal region of fanE, consisted of 251 amino acids with a 2 restriction enzyme sites Sau3AI and XmnI[57]. With a molecular mass of 22.9 and 31.5 kilodaltons for fanE and fanF respectively[82]. The FanF encode 333 amino acids[88] with two open reading frames reported, 999 bp [89] and 813 bp [57]. On the basis of DNA sequence homology, FanE is presumed to be a chaperone [90], FanF to FanH encode minor subunits are believed to be involved in pilus assembly and elongation [89].

Vaccines against K99 fimbrial ETEC

Since ETEC fimbriae are major colonizing factors in ETEC-associated diarrhea, studies focus on ETEC fimbriae as the primary target for vaccination against ETEC [91].

Early studies used purified *Escherichia coli* K99 pili as well as Formalin-killed whole cell bacterin as a vaccine against enterotoxigenic colibacillosis in suckling calves and piglets, This vaccine was safe and effective against K99 [92]. Other studies were conducted to examine the protecting effect on newborn calves born through vaccination of pregnant cows and heifers with the crude K99 extract prepared from an *Escherichia coli* K-12, K99 strain. Anti-K99 colostral antibodies were detected in the vaccinated group. Calves born by the vaccinated cows, when challenged orally with enterotoxic *E. coli* B41 (0101:K99+, ST+) at the time of first colostrum uptake, had no death occurred. In contrast all calves in the control group died [93].

K99 antigens were attempted to be expressed in plants as an edible vaccine candidate. The major K99 fimbrial subunit used as an edible subunit vaccine, where cytosol targeting with a synthetic version of FanC in soybean was developed and the extracted protein from the transgenic leaves develop a high antibody titer in mice [84]. Subcellular targeting of FanC to chloroplasts as edible transgenic plants was developed, but did not reach their full potential as a renewable source of oral vaccines [94].

Recently some researcher started to use a mucosal delivery system trying to elicit a better systemic and mucosal immune response. One group constructed a balanced lethal stabilized asd+ plasmid carrying the complete K99 operon, introduced into an attenuated *Salmonella typhimurium* to expresses *E. coli* K99 fimbriae, and found K99 fimbriae were effectively delivered to mucosal inductive sites for sustained elevation of IgA and IgG antibodies and for induction of protective [95]. Another group used a recombinant fusion proteins of pgsA and fimbriae protein of ETEC K99 to expose the fusion proteins on the surface of non-pathogenic strain *Lactobaccilus casei,* orally and intranasally immunized

BALB/c mice. They found immunized mice developed both systemic and mucosal immune responses, with high levels of serum immunoglobulin G and mucosal IgA against ETEC K99 [37]. It was also reported that a vaccine used *Lactobacillus casei* as a mucosal delivery to express K99 or K88 elicited high levels of mucosal IgA titers in mouse bronchoalveolar lavage fluids, intestinal fluids and prominent systemic IgG subclasses (IgG1, IgG2b, and IgG2a) responses in sera [96].

Bovine viral diarrhea virus

Viruses have two different strategies to produce infection. They can produce a persistent infection in individual animals or they use a hit and run strategy with a short duration of infection and rapid transfer to the next host [97]. Bovine viral diarrhea virus (BVDV) is one of the most important pathogens of cattle [9]. It causes significant economic losses worldwide. BVDV uses a persistent infection strategy to produce infection. It was first recognized in the United States in association with outbreaks of acute and fatal disease charterizied by diarrhea, high mortality rate and erosive lesions of the digestive tract [98, 99]. It infects domestic and wild ruminants worldwide [100]. BVDV infections ranged from clinically in apparent to severe disease involving one or more organ systems [99]. BVDV is a small positive stranded RNA virus. It belongs to the Falviviridae family and pestivirus group. It has two genotypes, the BVDV1 and BVDV2. BVDV is also unique in that in cell culture it exhibits two different biotypes, non-cytopathic (ncp) or cytopathic (cp) strains [100].

The ncp biotype of BVDV infections has the biggest impact on cattle during the breeding season or pregnancy[101]. If the infection occurs early in gestation (40-120 days of gestation) before the adaptive immune system matures, the ncp BVDV may

establish persistant infected animal (PI). The fetus may be born healthy but remains infected for life, and also remains immunotolerant to BVDV and remains a reservoir of the virus that can then infect healthy animals [9, 102]. Mucosal disease (MD) occurs when the PI animals acquire an infection with the cp-BVDV biotype, the combination of cp and non cp BVDV strains causes a MD form of BVDV, characterized by the widespread lesions in the gastrointestinal tract with diarrhea and respiratory symptoms [9].

BVDV genome

The BVDV has a positive single stranded RNA genome. It has a single ORF that is divided into different viral proteins, the structural and non structural proteins. The structural proteins are located at the 5` end, the non-structural proteins are located on the 3` end of the genome [9]. Four official species of genus Pestivirus have been identified, include BVDV-1, BVDV-2, classical swine fever (CSF) and border disease virus (BDV) [103]. BVDV1 and BVDV2 are two BVDV genotypes with non-cytopathic (ncp) and cytopathic (cp) biotypes [100]. The ncp strains are more common than the cp strains and the BVDV-1 genotype is more common than BVDV-2 genotype [104]. The cp   BVDV is able to produce a morphological changes in the tissue culture including vacuolation and cell death while the ncp is not producing any morphological changes in the tissue culture [102]. BVDV ORF encodes the following proteins from 5' to 3'. NH_2-N^{pro}-capsid-E^{rns}-E1-E2-P7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH [105, 106], this, single polyprotein is post-transitionally processed to produce the mature structural and non-structural proteins [107].

The structural proteins (capsid-E^{rns}-E1-E2) are incorporated into the virus structure or particle. The capsid or core protein encapsulate or coat and protect the genomic RNA[106]. The E^{rns} protein is a heavy glycosylated protein, while E1 and E2 are integral membrane protein [106]. A major part of humoral immune response and strongly neutralizing antibodies were identified against E2 glycoprotein, where the major antigenic determinants and the major part of the humoral immune response is directed to the N-terminus of the E2 glycoprotein [107-110]. That is why it is important to include the E2 glycoprotein in a BVDV vaccine. The 6 non-structural proteins are located in last two-thirds of the ORF and are involved in the viral replication[102]. N^{pro}, a non-structural and unique protein for pestiviruses, is an autoprotease, which sometimes cleaves the polyprotien after translation[106]. it blocks the interferone production by directing IRF3 (the interferon inducer in viral infected cells) proeosomal degradation [97]. The cleavage of NS2/3 is responsible for the production of cp effect in tissue culture, where the persistent infected immunocompetent animal combined with cp BVDV can develop mucosal disease [102].

BVDV vaccine

The glycoprotein E2, the most immunogenic protein of BVDV and elicits high titers of neutralizing antibodies after infection [110]. The virus transmission is blocked in the presence of neutralizing antibodies. Therefore, E2 constitutes an excellent antigen for vaccine candidates [111]. E2 protein of BVDV can be expressed with different expression systems. One study expressed the glycoprotein E2 subunit vaccine in the baculovirus expression system, and reported a prevention of transplacental transmission of virus beside protection of sheep fetus against homologous challenge [112]. Another

study used the baculovirus expression system for the production of a E2 glycoprotein vaccine or a vaccine that contained the structural polyproteins E0, E1, and E2 of BVDV. Both vaccines induced a solid humoral neutralizing immunity without complete protection in cattle [113].

Another group expressed the E2 protein of bovine viral diarrhea virus (BVDV) in fowlpox virus (rFPV/E2) and then immunized mice. The mice demonstrated both BVDV-specific humoral and cellular immune responses and the antibodies were able to neutralize BVDV [114]. Another group used three replication defective recombinant adenoviruses expressing the BVDV/E2. All three recombinant adenoviruses induced very strong BVDV specific antibody responses in a mouse model and produced a neutralizing antibody [115]. Another approach was to increase the E2 secretion [116] by intradermally immunizing mice with plasmid DNA encoding a truncated, secreted form of the BVDV E2 protein using four plasmids (pMASIA-gDsΔE2, pMASIA-tPAsΔE2, pSLKIA-gDsΔE2 and pSLKIA-tPAsΔE2). The pMASIA-tPAs_E2 elicited the strongest humoral and cellular immune responses and was identified as a candidate BVDV DNA vaccine.

Epitope vaccines

Epitope-based vaccine is an important approach to improve the immune response by isolation or optimization of specific components to induce the immune response against the pathogen [117]. In case of intracellular pathogens, as viral infection, it is important to identify putative T-cell epitopes through bioinformatics tools. The T cell epitopes play a role in the recognition and subsequent elimination of intracellular pathogens and improve the vaccine efficacy. Generally, epitope is the antigenic determinant or it is part of a macromolecule that is recognized by the immune system,

specifically by antibodies, or B or T cells. Computer based algorithms can detect antigenic peptide domains of a given protein which are likely to induce cellular immune response [118].

Previously described ETEC and BVDV vaccines based on the entire pathogen organism, or proteins. While the epitope vaccines induce protective immune using selective epitopes without a risk of induction of the disease. So that an epitope based vaccine is safe, which is able to stimulate an effective specific immune response and avoid undesirable effects. It is composed of MHC-I and/or MHC-II epitopes which are required for stimulation of an effective immune response. There might be some limitation due to the use of small molecules which might be low immunogenic, so that the coupling with another protein using recombinant DNA technology or the administration of an appropriate adjuvant could enhance immunogenicity of epitopes.

There are over 150 licensed biological products for BVDV control in United States, but none of them conclusively identified to stimulate a protective immune response against BVDV [119]. Identification of epitopes on a putative viral envelope glycoprotein, gp53 could be a good approach for BVDV control through viral neutralization. Neutralizing antibodies are considered an effective line of defense as they can intercept a virus before it attaches to its target cell and, thus, inactivate it [120]. Although there is no epitope vaccines developed for commercial use, several studies targeting specific immunogenic epitopes in vaccine design showed promising results for disease control. This might be considered an alternative approach for vaccine development. It was reported that an epitope based broad-spectrum influenza vaccine, which included epitopes from a majority of influenza virus strains, activated both the

humoral and cellular immune response [121]. Another study showed that a vaccine composed of CD4^{+} T cell epitopes from *H. pylori* adhesin A (HpaA), urease B (UreB) and cytotoxin-associated gene A product (CagA) with Th1 adjuvants elicited strong local and systemic Th1-biased immune response against H. pylori in a mouse model [122]. In the influenza virus vaccine, two surface protein antigens, the hemagglutinin and neuraminidase, were considered to be included in the vaccine [15]. Here, we designed a multiepitope vaccine which consists of K99 FanC, a pSTa toxoid, E2 B and T-cell epitopes for a broad protection against ETEC and BVDV.

Objectives of the study

The enterotoxigenic *Escherichia coli* (ETEC) strains expressing K99 (F5) fimbriae are the major cause of diarrhea in calves. The bovine viral diarrhea virus (BVDV) affects cattle of all ages. The K99 and BVDV can infect young calves causing severe diarrheal disease and huge economic losses. Particularly, it is makes sense to use a vaccine to protect calve against both pathogens and might also cattle at other ages. Previous vaccine candidates against *Escherichia coli* K99 consisted of single protein targeting FanC the major K99 subunit or the whole cell bacterin. Most of the bovine viral diarrhea vaccines were directed against E2 glycoprotien. No previous study combines K99 and BVDV in a single vaccine for providing a protection against both pathogens.

A vaccine for protection against both ETEC & BVDV infections has not been developed. A vaccine candidate based on K99 fimbria might be a good approach. K99 colonization and the STa enterotoxin are the major virulence attribute of bovine ETEC. The E2 glycoprotein has a major role in BVDV entry and attachment in cattle. We decided to use a novel approach to include some epitopes against those virulence factors.

Free online software was used to detect B and T-cell epitopes of BVDV to be embeded to the K99 major fimbriae, fanC. The pSTa$_{12}$ toxoid also included. It is well known that B-cell epitopes play an important role in vaccine design. We select our BVDV B cell epitope from E2, where the amino acid sequence 71–74 considered immunodominant region and contain the common neutralizing epitopes on the BVDV-E2 (gp53) protein [108, 124].

Our research objective on chapter two was to construct a novel subunit vaccine candidate for the prevention of bovine pathogenic *E. coli* & BVDV infections in a murine model. FanC would be used as a backbone, and the pSTa toxoid and the E2 B and T-cell epitopes would be embedded in to FanC. This fusion antigen would provide anti-adhesion, anti-toxin, and anti-BVDV immunity, and potentially to be developed as a subunit vaccine against K99 ETEC and BVDV in cattle.

Chapter Two

Immunogenicity study of a novel chimeric fanC subunit antigen with embedded pSTa

toxoid of enterotoxigenic *Escherichia coli* (ETEC) and BVDV E2 B and T cell epitopes

of bovine viral diarrhea virus (BVDV)

Abstract

Diarrhea is the most important problems in cattle. Enterotoxigenic *Escherichia coli* (ETEC) strains expressing K99 (F5) fimbriae and heat-stable type I (STa) enterotoxin are the major cause of diarrhea in calves, while the bovine viral diarrhea virus (BVDV) is the major cause of diarrhea in all ages of cattle. However, there is no single vaccine combined those two diseases. The conventional vaccines, including a single antigen from either fimbriae or enterotoxins, are not effective. It is important to develop a prevention strategy that can protect against diarrhea in calves. The goal of the work presented here was to create a multivalent vaccine that protects calves against both pathogens. The K99 adhesin and STa enterotoxin are the major virulence determinants for ETEC, while the E2 glycoprotein is the most immunogenic protein of BVDV. We hypothesized that a novel multivalent vaccine consisting of FanC, the major fimbrial subunit of K99, STa and BVDV E2 antigens will induce broad protective immunity. We constructed a genetic fusion protein using the K99 major subunit FanC as a carrier of STa, B-cell and T-cell epitopes of the BVDV E2 glycoprotein (with STa & BVDV epitopes were embedded in the FanC major subunit). FanC exposed non conserved epitopes was replaced by STa and BVDV E2 epitopes. This fusion protein was tested in a murine model for safety, immunogenicity and to examine vaccine potency. Expression of the chimeric K99 FanC fimbriae, STa and BVDV-E2 antigens in these constructs was

expressed in a non-pathogenic *E. coli* strain and purified to be used in our study. Female BALB/C mouse 42-49 days immunized intraperitoneal (i.p.) with 200 μg purified fusion protein in an equal volume of adjuvant. The immunized mice developed strong humoral immune responses, anti-K99, anti-STa, and anti-BVDV antibodies. The antibodies from mouse sera samples were able to block ETEC K99 strain adhesion to INT-407 and IPEC-J1 cells on a bacteria adherence blocking assay. Anti-STa antibodies were protective in vitro in cyclic GMP EIA as they could reduce the toxicity of STa toxin to T84 cells. Anti-BVDV antibodies were able to prevent the BVDV NADL starin cytopathic effect in MDBK cells. This suggested that the K99 FanC fimbria can be served as a platform to express enterotoxin and BVDV antigens to induce protective antibodies. Besides, the novel fusion can be an ideal antigen for providing a broadly protective vaccine against diarrhea in cattle.

Introduction

Diarrhea cause large economic losses especially among young animals, it might be a principal or secondary symptom of different diseases [2]. Enterotoxigenic *Escherichia coli* (ETEC) is the major bacterial enteric diseases causing diarrhea in calves [8]. Meanwhile, bovine viral diarrhea virus (BVDV) consider the major problems causing diarrhea in calves as it leads to widespread lesions in the gastrointestinal tract and development of mucosal disease [9]. Two major virulence factors should be included in ETEC infection and pathogenesis, the adhesions or fimbrial colonization to the small intestine and secretion of the enterotoxins. STa is the major enterotoxin secreted by K99 ETEC causing diarrhea. E2 glycoprotein has a major role in BVDV entry and attachment in cattle plus the production of neutralizing antibodies, so that two E2 epitopes included

in our antigen (B and T-cell epitopes) from the E2 glycoprotein. No standard vaccine providing a protection against ETEC & BVDV was developed in a single product. So that any effective vaccine against bovine ETEC, including the major virulence factors with E2 glycoprotein, is suspected to provide a protection against ETEC and BVDV. In this study, the non conserved exposed epitopes of the FanC major subunit was replaced by the three selected epitopes, the pSTa, E2 B-cell & T-cell epitopes to express fanC, STa and E2 antigen in a single protein. The safety and immunogenicity of our antigen was tested in murine model and showed that our antigen can be used for potential application and vaccine development against bovine diarrhea caused by ETEC and BVDV.

Materials and Methods:

Bacterial strains and plasmids: The plasmid K99, which contains the entire K99 fimbrial gene cluster (FanA to FanH) [1, 73] in pBR322, was provided as a gift from Dr. R. E. Isaacson, at the Veterinary and Biomedical Sciences Department, University of Minnesota, Minnesota, USA [1]. This 1297 strain which carries the K99 operon (figure 1) was used for fimbrial chimera construction and pK99 fimbrial extraction (figure 2). Different primers were used for the construction of chimeric FanC (the major structural-subunit) of K99 fimbria. All strains and plasmids used in our study are listed in (table 3). Plasmid pET28a (Novagen) was used to clone and express the recombinant *FanC* gene with the embedded $pSTa_{12}$, BVDV E2 B and T cell epitopes. Plasmid pMAL-p5X (New England Biolabs) was used to clone the BVDV-E2 gene for production of MBP (maltose binding protein)- E2 fusion as coating antigens for detection of BVDV antibodies using ELISAs.

pIX12 (7.15 kb Bam HI insert)
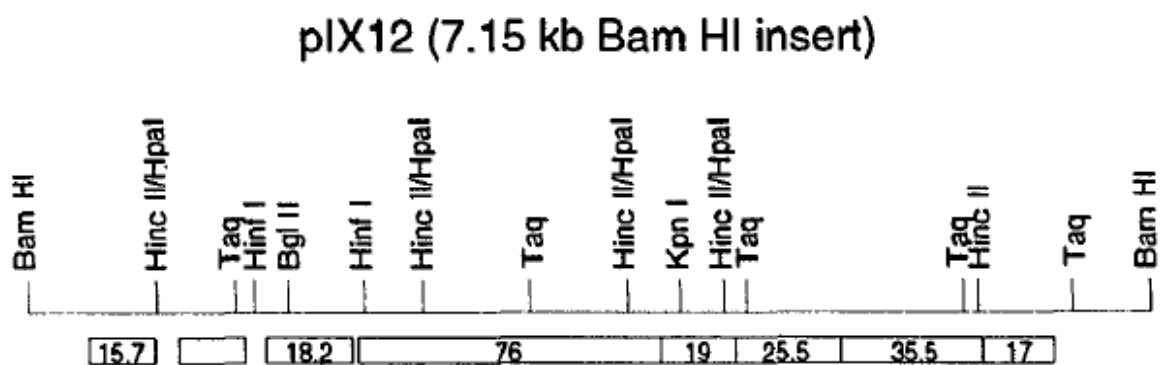
Fig.1. Illustration of K99 fanC operon in recombinant *E. coli* strain 1297. Restriction mapping, size of various genes are shown in the diagram [1].
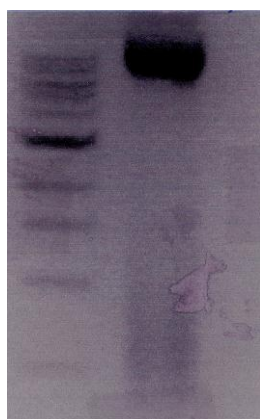
Fig.2. Gel electrophoresis of the K99 plasmid. pK99 extraction in 1% agarose gel with 1kb marker. The expected size of the pK99 plasmid is 11.5 kDa.

Prediction of FanC non conserved exposed peptide domains:

The ConSurf server software was used for identification of antigenic regions in the protein. This program was used to detect the FanC for non-conserved but surface-exposed epitopes http://consurf.tau.ac.il/ [125, 126]. These predicated epitopes were the targets to be replaced with pSTa$_{12}$ and BVDV-E2 epitopes. The conserved and the most antigenic epitopes were retained for induction of anti-K99 immunity (figure 3).

Inserted toxoid ST$_{ap12F}$ and two BVDV epitopes:

Porcine *Escherichia coli* ST$_{ap12F}$ toxoid was inserted in FanC for the FanC-STa chimeria. The selected pSTa$_{12}$ has a mutation in Pro12. Previous studies showed that Pro12 mutation is similar to other STa mutants showing a reduction in STa toxicity [127, 128]. The inserted ST$_{ap12F}$ sequence is (NTFYCCELCCNFACAGC). Since B-cell epitopes plays an important role in vaccine design and the T cell epitopes plays an important role in controlling the viral infection, two E2 epitopes from BVDV type-1 NADL strain envelope glycoprotein E2 were included: the most antigenic B-cell epitope and the most antigenic T cell epitope. Prediction analysis showed that the peptide 71–74 represented an immunodominant region and a common neutralizing epitopes on the BVDV-E2 (gp53) protein [108, 124]. The BVDV E2 B-cell epitope was encoded from nucleotides, *cat aca aga gcc ttg ccg acc agt gtg gta ttc* which translated to11 amino acids(^{70}HTRALPTSVVF^{80}). T-cell epitopes were predicted based on MHC class I predication. The BVDV E2 T cell epitope was drived from nucleotide fragment, *gac caa cta cta tac aaa ggg ggc tct* which translated to 9 amino acids (^{193}DQLLYKGGS^{201}). Amino acid residues 886 and 888 of the E2 T cell epitope (underlined), were reported as

positively selected sites of BVDV type1 on the E2 gene [129]. All epitopes were

confirmed using epitope selection software.
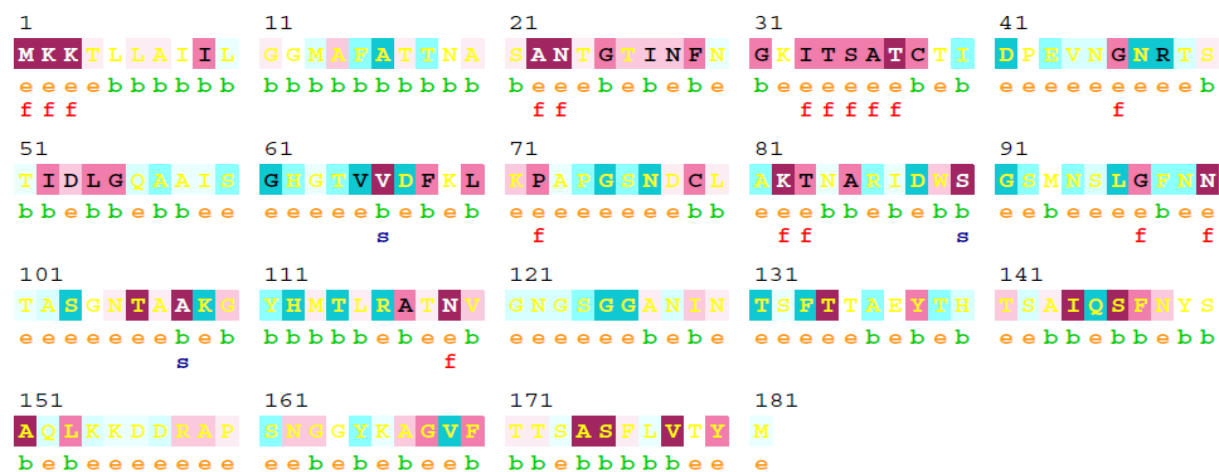
Fig.3. Predication of antigenic regions in FanC protein (the K99 major subunit) which composed of 181 amino acids. Detected non conserved exposed amino acids were replaced with pSTa$_{12}$ and BVDV E2 epitopes.

Epitope prediction with computer-based programs:

Different software was used to predict BVDV E2 B and T cell epitopes. BVDV-NADL

E2 glycoprotein amino acid sequence (accession NP_040937.1) was directly used in

bioinformatics prediction software, then selection of the most common epitopes that have

been repeated in different software. http://tools.immuneepitope.org/main/index.html

[130], http://imtech.res.in/raghava//abcpred/index.html [131]

http://ailab.cs.iastate.edu/bcpred/index.html [132] and

http://www.imtech.res.in/cgibin/propred1/propred1.pl [133].

Construction of the FanC chimeric gene:

The *FanC* gene was amplified in PCR with pK99 plasmid as DNA template and

specifically designed PCR primers (table.1). ConSurf software predicated peptides 91-

107, 116-126 and 154-162 of FanC as surfaced-exposed but less antigenic. These three

fragments were removed through different PCR reactions and replaced with the

Escherichia coli pSTa$_{12}$ toxoid, and E2 B and T cell epitopes respectively using different

primers (table 1). Splice overlapping extension (SOE) PCR was used to construct the

chimeric FanC .PCRs were performed with a PTC-100 thermal cycler (Bio-Rad,

Hercules, CA) in a 50µl reaction mixture containing 1X *Pfu* DNA polymerase buffer

(with Mg2$^+$), 200 nM deoxynucleoside triphosphate (dNTP), forward and reverse primers

at 0.5 µM each, and 1 U Pfu DNA polymerase (Stratagene, La Jolla, CA). SOE PCR was

performed in a reaction mixture containing 1X *Pfu* DNA polymerase buffer (with Mg2$^+$),

200 nM dNTP, 20µl each of the 5`- and 3`- end purified PCR products, 1 U of *Pfu*

polymerase, and 0.5 U Taq DNA polymerase (Applied Biosystems, Foster City, CA).

Amplified products were separated by 1-1.5% agarose (FMC Bioproducts, Rockland,

MA) gel electrophoresis and purified using a QIAquick Gel Extraction Kit by following the manufacturer's instruction (QIAGEN, Valencia, CA).

Cloning and expression of the chimeric gene:

Amplified FanC chimeric gene product was digested with the unique restriction enzymes and the cloning vector was digested with the same enzymes. Restriction mapping software http://tools.neb.com/NEBcutter2/ was used for selection of the unique restriction sites. The purified PCR products and expression vector pET28a were digested with Nhe1 and EagI restriction enzymes (New England Biolab, Ipswich, MA) respectively. Two microliters of ligation products were used for transformation of *E. coli* BL21 competent cells under a standard procedures [134]. The digested gene products and vectors were purified and then ligated with T4 DNA ligase (New England BioLab). Two microliters of T4 ligated products were introduced to competent cells by electroporation. Positive colonies were initially screened by PCR then sequenced to ensure that the cloned genes were inserted in reading frame. The positive colony was selected for protein expression (figure 4).

Preparation of the 6×His-tagged chimeric protein:

Positive transformed cells grew in Luria Broth (LB) overnight at 37ºC in the presence of kanamycin, to an optical density at 600 nm (OD600) of 0.5, then pET28a recombinant vector were induced using 1mM isopropyl-ß-$_D$-thiogalactopyranoside (IPTG) for an additional 4 h of culture. Inclusion bodies (IBs) were isolated using BugBuster Reagent BBR (Novagen) following the manufacturer's instructions. Simply, the overnight grown culture was centrifuged 5000 Xg for 10 minutes at 4°C, pellets were collected for total protein preparation using B-PER reagent (in phosphate buffer) (Pierce, Rockford, IL).

The pellet was dissolved in B-PER, by vortex or pipeting up and down, then centrifuged 15000 Xg for 15 minutes at 4°C. The supernatant was collected and labeled as soluble protein. The pellet was resuspended with 1:10 diluted B-PER in PBS then mixed with lysozyme 10mg/ml in B-PER then centrifuged 15000 Xg for 15 minutes at 4°C. Finally the pellet was resuspended two more time with diluted B-PER in PBS and the inclusion bodies were collected in PBS.

Extraction and refolding of fusion protein:

Purification of the inclusion bodies was done through Ni-NTA Beads for our 6x his-tagged protein. The beads were washed; the protein was eluted in small volumes of buffer and confirmed by Western blotting and Coomassie blue staining. Dialysis of the purified protein against PBS overnight at 4°C. Extracted inclusion bodies were refolded using a Novagen refolding kit (Merck KGaA, Darmstadt, Germany). Briefly, the sample was collected from the overnight dialysis in PBS and spin at 4200 rpm at 4°C for 30 minutes, then the solubilization buffer was added to the pellet and dissolved gently by gentle mixing or pipeting up and down. The solubilized protein was refolded by the dialysis of the protein against refolding buffer. In summary, the fusion protein extracted in the format of inclusion bodies, solubilized and refolded before being used in mouse immunization.

Table.1:

PCR primers designed to construct chimeric fanC gene. Underlined nucleotide represents

the restriction enzyme sites, italic nucleotides are the inserted epitopes.

Primer	Sequence (5`- 3`)
FanC-NheI-F2	5`-ATGATG<u>GCTAGC</u>ACACTCCTAGCTATTATCTTAGGT -3`
FanC-EagI-R	5`-TCATCGATA<u>CGGCCG</u>CAATGTAA-3`

STa12-fanC91-F

5`- GAACTTTGTAATTTTGCCTGTGCTGGATGTGGAAATACTGCTGCTAAAGGATACCAT-3`

STa12-fanC91-R

5`-GGCAAAATTACAACAAAGTTCACAGCAGTAAAATGTGTTGTTAGACCAGTCAATACGAGC-3`

BVDV-fanC116-F

5`-GCCTTGCCGACCAGTGTGGTATTCGCTAATATTAATACTTCATTCACTACG-3`

BVDV-fanC116-R

5`-ACTGGTCGGCAAGGCTCTTGTATGCAAAGTCATATGGTATCCTTTAGCAGC-3`

BVDV-fanC154-F

5`-*CTACTATACAAAGGGGGCTCT*GGTGGATATAAAGCTGGCGTATT-3`

BVDV-fanC154-R

5`-GCCCCCTTTGTATAGTAGTTGGTCCAGCTGGGCTGAATAGTTAAATGACT-3`
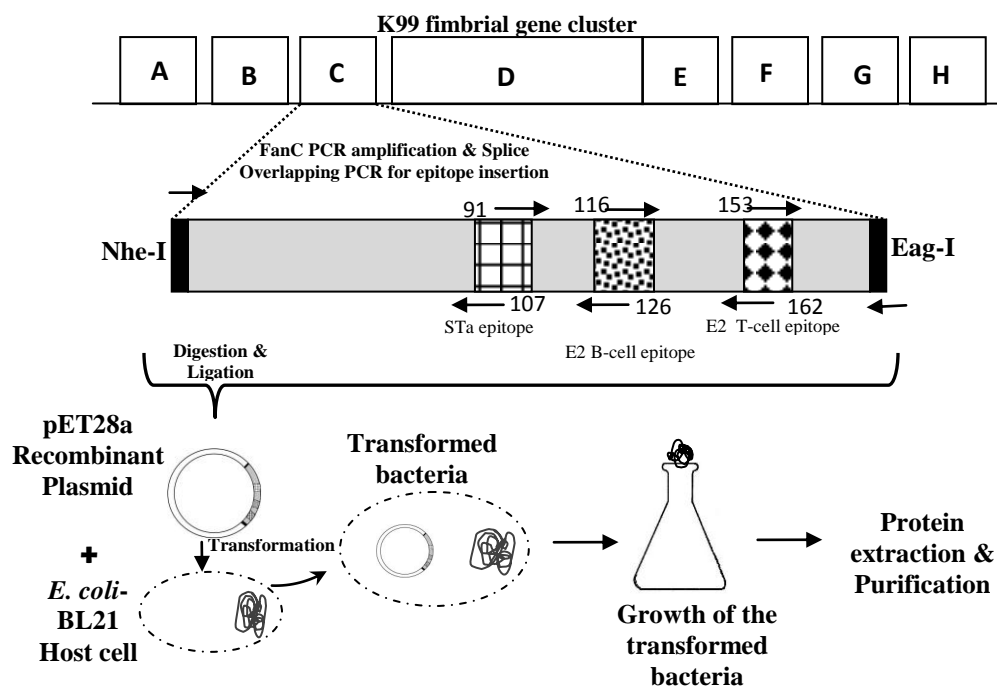
Fig.4. Scheme of construction of chimeric FanC-STa-E2 protein. Arrows represent the primers used to insert STa and E2 epitopes. Transformation in *E coli* BL21 competent cell & the protein was extracted and purified.

Detection of fusion protein by SDS-PAGE& Western blotting:

Protein was examined by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The proteins were separated according to the molecular weight and visualized. Thirty microliters of prepared proteins were used for detection of the target protein in a standard SDS-PAGE and immunoblot assay method. After electrophoresis, gels were either stained with Coomassie blue or transferred to a nitrocellulose membrane. The blot was blocked with 0.5% milk in PBST /1 hr at RT. The membrane was incubated with one of these antibodies: anti-K99 (1:1000), anti-STa (1:2500) or anti-BVDV antibodies (1:1000). The blotted membrane was overnight incubated at 4°C of with the primary antibody. Multiple antibodies were used to ensure that the fusion proteins with the STa and E2 embedded epitopes. Membrane was washed with PBST three times and incubated with secondary antibody (HRP)-conjugated goat anti-mouse IgG (1:2500) or IgA (1:1000) for 1 hr at RT. After this, it washed with PBST three times and PBS once. The bloated protein was examined and detected on Odyssey Infrared Imaging System (figure 5).
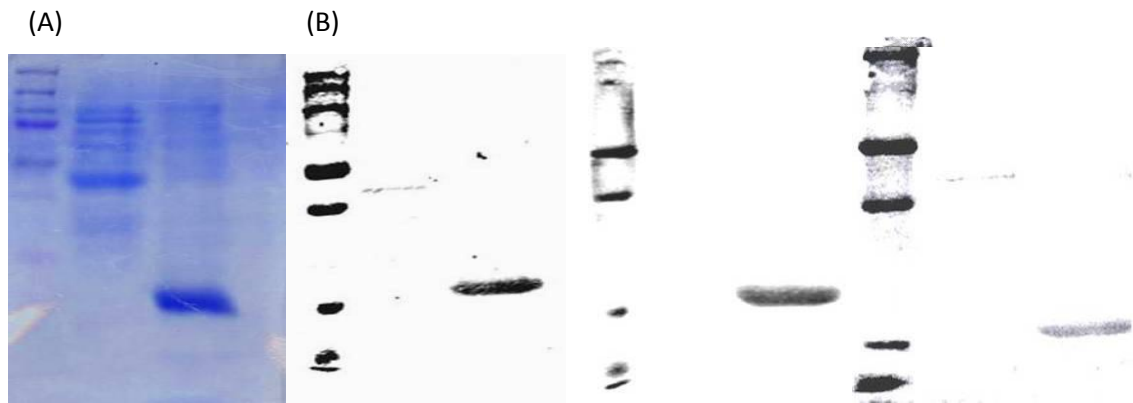
Fig.5. Detection & characterization of the FanC-STa-E2 fusion protein. (A)Coomassie blue staining in SDS 12% polyacralymide gel. (B)Western blotting detection of the fusion protein using one of these: anti-K99 polyclonal antibodies 1:1000, anti-STa monoclonal antibodies 1:2500 or anti-BVDV-NADL strain polyclonal antibodies 1:1000. Band with a molecular mass of 22.5 kDa

K99 fimbrial extraction:

E. coli K99 fimbriae were heat extracted based on a described method [135]. The technique was similar to the one used to isolate K88 fimbriae but with some modification. Briefly, *Escherichia coli* K99 was grown on fresh sheep blood agar for 18 h at 37 °C and harvested in PBS. Harvested bacteria were suspended in PBS, and bacterial suspension was gently shaked at 65 °C for 40 minutes. While it still warm, the mixture was poured into a blender and blended for 2-3 minutes. Bacteria debris were removed by centrifugation 15000 xg for 30 minutes. The supernatant was poured into a dialysis tube and kept overnight at 4°C. The pH of the supernatant fluid was adjusted to 4.0 by using dilute acetic acid 2.5% with continuous stirring for 10 minutes at 4°C or on ice. Crude antigen was found on the supernatant and Western blot analysis was confirmed the K99 fimbrial protein using anti-K99 polyclonal antibodies 1:1000 (figure 6). Lawry protein assay, measures the protein concentration. The purified fimbrial antigen 500ng was used as a coating antigen for ELISA.
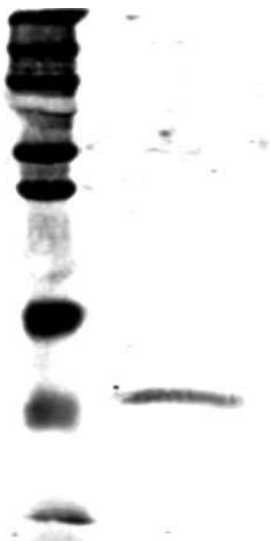
Fig.6. Western blotting detection of K99 fimbrial major subunit FanC using anti-K99 polyclonal antibodies 1:1000.

Construction of BVDV-E2 antigen

BVDV-E2 antigen construction was described (figure 7). Plasmid pMAL-p5X (New England Biolabs) was used to clone the BVDV-E2 gene for MBP-E2 fusion protein. This protein was used as the coating antigen for detection of BVDV antibodies, from the animal study, by using the ELISA technique. Amplification of the BVDV E2 glycoprotein gene was done in a RT-PCR reaction using different forward and reverse primers for each construct, including the unique restriction sites listed in (table.2). RT-PCR rcreation is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA. Amplified E2 gene product was digested with unique restriction enzymes detected by the restriction mapping software http://tools.neb.com/NEBcutter2/. DNA fragment of purified PCR products and plasmid pMAL-p5X were digested with Nde1 and BamH1 restriction enzymes (New England Biolab, Ipswich, MA). Two microliters of ligation products were used for transformation into a non pathogenic *E. coli* BL21 competent cells under a standard procedures [134]. Digested gene products and pMAL-p5X vector were purified and ligated with T4 DNA ligase (New England BioLab). Two microliters of T4 ligated products were introduced to competent cells by electroporation. Positive colonies were screened by PCR then sequenced to ensure that the cloned genes were inserted in reading frame. Successful positive transformed cells were allowed to grow in small bacterial culture in Luria Broth (LB) of 10 ml overnight culture at 37ºC in the presence of ampicillin. Then transferred to 1 liter of LB broth and induced with 1mM IPTG at OD600) of 0.5 for an additional 4 h of culture. Protein was purified according to the pMAL protein fusion & purification system

(Newengland Biolabs). The mannose binding protein (MBP) was attached to our protein; we were not able to cleave our protein from MBP using the standard protocol. The fusion protein was confirmed by Western blotting using anti-BVDV polyclonal antibodies. The protein concentration was measured using Lawry protein assay. The purified MBP-E2 protein 500 ng was used as a coating antigen for ELISA to test the anti-BVDV antibodies from mouse study.

Table.2: PCR primers used to construct BVDV NADL E2 gene. Underlined is the restriction enzyme site.

Primer	Sequence (5`- 3`)
BVD-E2-Nde1-F	5`- ATTTCA<u>CATATG</u>CACTTGGATTGAAAACCTGAA-3`
BVD-E2-BamH1-R	5`- TCTGTA<u>GGA TCC</u>AGGCATAGGTCCGAGTTTGGT-3`
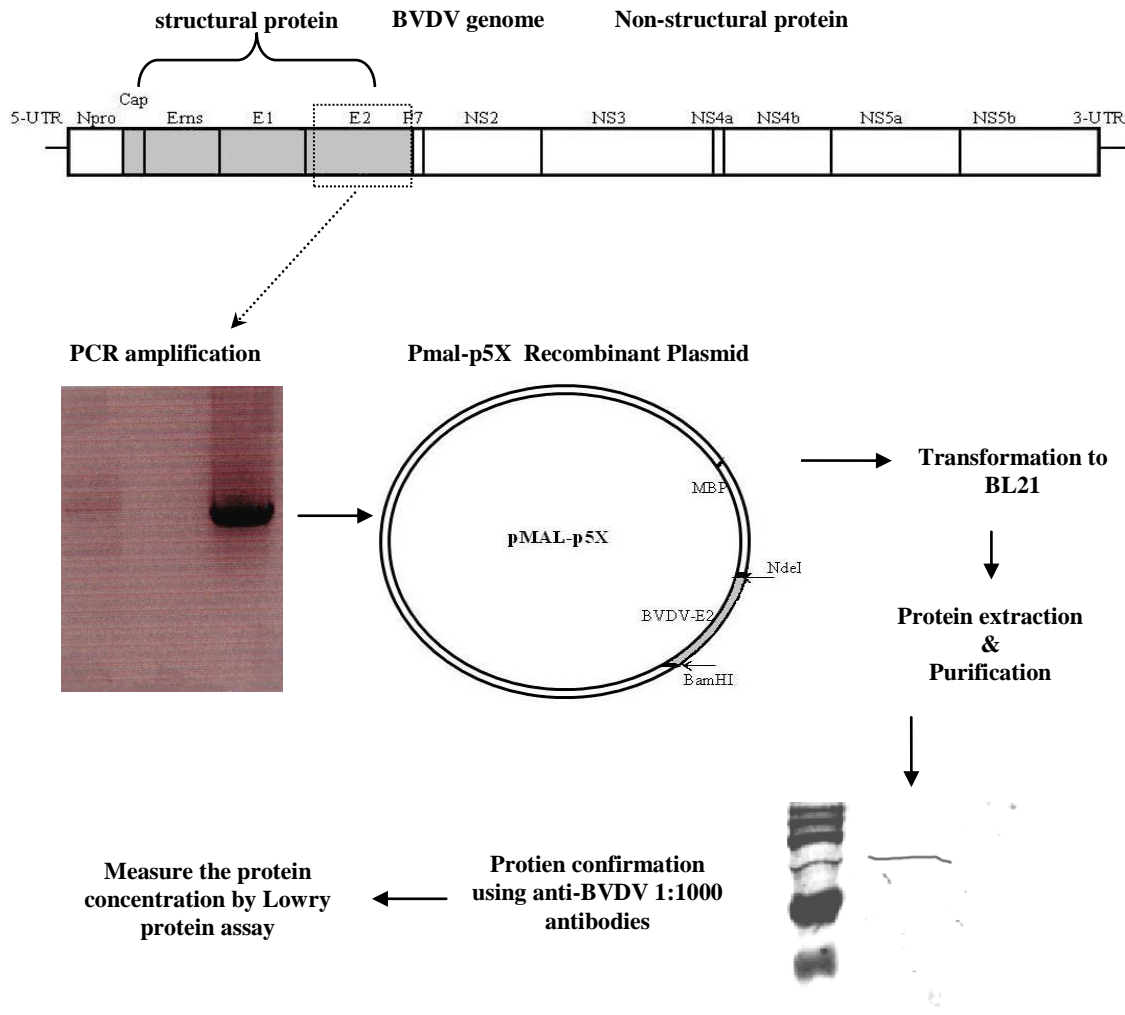
Fig.7. Construction of MBP-E2. BVDV genome with structural protein (gray), & non-structural protein (white). The strain used was the cytopathic BVDV NADL. BVDV-E2 glycoprotein amplified the PCR product by using BVDV-E2-Nde1-F & BVDV-E2-BamH1-R primers in 1% agarose gel with 100 base pair marker. Amplified band with a molecular mass of 1000 bp. PCR product was ligated to pMAL-p5X plasmid for the expression of MBP-E2 protein. MBP-E2 of a molecular mass of 75kDa verified with anti-BVDV polyclonal antibodies 1:1000.

Mouse immunization:

Sixteen female BALB/C mouse 42-49 days was used to test the subunit vaccine. Mice were randomly divided into two groups, eight mice immunized intraperitoneal (i.p.) with 200 µg purified fusion protein that were diluted in PBS and emulsified in an equal volume of Freund's complete adjuvant (FCA) (Sigma-Aldrich, MO), followed by two booster injections on day 14 and 28 post primary immunization of purified proteins that were diluted in PBS and emulsified in Freund's incomplete adjuvant (FIA). Eight mice without immunization served as a negative control group and received the same adjuvant in PBS. Two booster injection doses were used at biweekly intervals (figure 8). All mice were sacrificed two weeks after the second booster injection. Blood & fecal samples were weekly collected. Blood was allowed to clot for collection of serum samples which stored at −80°C until use. All animal studies in this project complied with the Animal Welfare Act, followed the Guide for the Care and Use of Laboratory Animals, approved and supervised by South Dakota State University's Institutional Animal Care and Use committee.
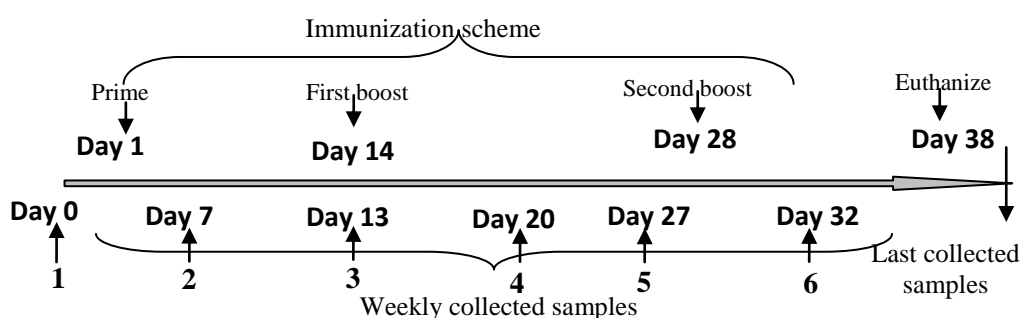
Fig.8. Mouse immunization scheme. Purified chimeric protein 200 µg injected IP in a three doses with two weeks intervals (indicated in the upper arrows). Serum and fecal samples were collected on a weekly interval (1-6 weeks) or (0-32 days) indicated in the lower arrows. Last collected samples were used for antibody titration, BVDV& STa neutralization assay.

Anti-mouse antibody titration:

Serum and fecal samples were examined for anti-K99, anti-STa and anti-BVDV antibodies. Purified ETEC K99 fimbria, STa ovalbumin conjugates and BVDV-NADL-E2 protein were used as antigens in enzyme-linked immunosorbent assays (ELISAs) to titrate anti-K99, anti-STa, and anti-BVDV antibodies, respectively. Five hundred nanograms of K99 fimbrial antigen or BVDV-NADL-E2 protein was used to coat each well of an immunolon[tm] 2HB ELISA plate (ImmunoChemistry Technologies, LLC, MN) to titrate anti-K99, anti-BVDV antibodies respectively in a standard ELISA. For anti-STa antibody titration, we coated each well of a Costar plate (Corning Inc., Corning, NY) with 10 ng of STa ovalbumin conjugate. Plates were coated with the selected antigens and kept at 4°C overnight. Double fold serial dilution of mouse serum samples starting from (1:640) or fecal samples supernatant starting from (1:10) were used as the primary antibodies. Meanwhile horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:2500) and IgA (1:1000) were used as the secondary antibodies. The optical density OD at 405 nm was measured after 30 min of development in peroxidase substrates (KPL, Gaithersburg, MD). The titers were determined by the log10 value of the highest dilution with an OD 0.3 after subtraction of the background. The error bars were calculated with the optical density (OD) values of triplicate wells of a specimen.

K99 adherence inhibition assay

K99 fimbrial ETEC and two different cell lines were used in adherence inhibition assays [136]. It has been reported that porcine intestinal epithelial cell line (IPEC-J1) and human Caucasian embryonic intestine cell line (INT-407) have the ability to bind to ETEC-K99 bacteria in vitro [137]. IPEC-J1 1×10^5 and 5×10^5 INT-407 were seeded into

each well of a 24-well tissue culture plate, maintained in a humidified atmosphere of 5%

CO_2 at 37ºC and suspended with DMEM/10% FBS or MEM/10%FBS

(GIBCO/Invitrogen) respectively. The tissue culture plate were dislodged by 100μl

0.25% trypsin at 37 ºC in 5% CO_2, suspended with same medium and counted under a

microscope. The subcultivation ratio was set to 1:3. ETEC-K99 bacteria were cultured

overnight on LB blood agar plates, harvested next day with swabs and suspended in PBS.

The multiplicity of infection (MOI) ratio was five bacteria per cell. One hundred

microliters of a bacterial cell suspension was mixed with 20μl mouse serum or fecal

samples and incubated at room temperature for 1 h on a shaker at 50 rpm. The mixture

was added to cells and incubated at 37°C in 5% CO_2 for 1 h. Cells were washed three

times with PBS and dislodged with 0.25% trypsin at 37°C in 5% CO_2 for 30 min,

collected by centrifugation (15,000 Xg for 10 min) and resuspended in 1 ml PBS. The

resuspension was serially diluted (10^2, 10^3, and 10^4), spread on LB agar plates, and

cultured at 37°C overnight. Colonies were counted, and adherence was calculated as the

number of CFU/ml. The error bars were calculated with CFU/ml of triplicate wells of a

specimen.

BVDV neutralization assay

Neutralizing antibody induced by E2 glycoprotein after infection or vaccination was

considered the main protective mediator against BVDV infection [138]. Viral

neutralization test was done [139] with some modification. The neutralization assay was

done using the serum or fecal samples antibodies, utilizing Madin Darby Bovine kidney

(MDBK) cells with 10% fetal bovine serum (FBS) as a supplementary medium and the

cytopathic NADL strain (ATCC VR-534). Briefly, 2.5 $X10^5$ MDBK cells (ATCC CCL-

22) were seeded to a 96 well tissue culture plate. The BVDV-free MDBK cell line was grown in MEM containing 20 µg/ml gentamycin and 10% FBS. Two-fold serial diluted samples were pooled starting with 1:40 dilution to 1:5120. Fecal sample supernatant or heat-inactivated serum (56°C/ 30 minutes) was mixed with equal volumes of virus containing 0.1 median tissue culture infectious doses (TCID50) and incubated for 1 h at 37°C in a 5% CO_2 incubator. The mixture was added to BVDV-free MDBK cells in 96 well tissue culture plate and kept for 3 days at 37°C in a 5% CO_2 incubator. All experiments were performed in triplicate and the neutralization data were referred to the non-immune serum. The ability of the tested sample to reduce the number of viral cytopathic effect was assayed in monolayers of MDBK cells. The highest dilution of serum which gave complete neutralization of virus, as determined by the absence of cytopathic effect (CPE) was recorded as the SN titer.

STa neutralization assay

Neutralizing activity of the STa toxin through the antibodies in mouse serum and fecal samples was examined. The human colon carcinoma cell lineT84 cells (ATCC CCL-248) was utilized and maintained in a humidified atmosphere of 5% CO_2 at 37 ºC and cultured in 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (Invitrogen, CA) supplemented with 5% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, MA), (the subcultivation ratio was 1:3). Cells were seeded in a 12-well tissue culture plate. Cyclic GMP (cGMP) enzyme immunoassay kit (Assay Design, Ann Arbor, MI) was used [136]. Briefly, $2X10^5$ T84 cells (greater than 85% confluence) were seeded into each well. The spent medium in the plate was removed, and the plate was washed twice with magnesium and calcium free saline (GKN, 1 mg/ml

glucose, 0.4 mg/ml potassium chloride, and 8 mg/ml sodium chloride). Cells were incubated with 700 µl DMEM/F12 medium with 1mM isobutylmethylxanthine (IBMX) at 37 ºC in 5% CO_2 for 45 min. Two ng of STa toxin (diluted in 150 µl of DMEM/F12) was incubated with 150µl of a mouse serum or fecal sample (1:10 dilution in DMEM/F12, in triplicate) at room temperature for 1 hr, then the mixture (150 µl of STa toxin dilution plus 150 µl of the serum or fecal sample dilution) was added to each well, and the plate was further incubated with IBMX-pretreated cells for additional 2-4 hours at 37ºC in 5% CO_2.The plate was washed twice with GKN, and, the cells were lysed with 0.1 M HCl (300 µl per well) HCl containing 0.5% Triton-X100 (Sigma-Aldrich, MO) and neutralized with 0.1 M NaOH. Cell lysates were collected by centrifugation at 660 Xg for 10 min at room temperature. The supernatant was tested for intracellular cGMP levels by following the manufacturer's protocol (Assay Designs, Inc., Ann Arbor, MI).

Construction of holotoxin like vaccine:

Plasmid pBR322 (Promega, Madison, WI) was used to clone and express the recombinant chimeric fanC gene expressing mutated STa gene, and bovine viral diarrhea B and T-cell epitopes. Plasmids and strains used are listed in supplementary table-1. The plasmid p9196 was used as a template for the chimeric fanC gene and the plasmid 9207 was used to amplify the holotoxin like structure. Different primers were used (supplemtary table). Amplified products were separated by 1% agarose gel electrophoresis and purified using a QIAquick Gel Extraction Kit by following the manufacturer's instruction (QIAGEN, Valencia, CA). Two different constructs were designed. The first construct (9300) contains fanC promoter, ribosomal binding site and signal peptides with HindIII/EagI restriction sites. The second construct (9205) includes

the p1& p2 prokaryotic promoter for pBR322 plasmid using with Nhe1/EagI restriction sites. The reason for constructing two different plasmids was to try to compare the protein expression in both constructs. Purified PCR products and plasmid pBR322 were digested with the unique restriction enzymes. Ligation products were used for transformation into a non pathogenic *E. coli* DH5α competent cells under a standard procedures [134]. Positive colonies were initially screened by PCR and sequenced to ensure that the cloned genes were inserted in correct reading frame. Successful positive transformed cells were grown in Luria Broth (LB) overnight with shaking (200 rpm) at 37ºC in the presence of ampicillin. The bacterial culture was centrifuged at 5,000 Xg for 10 min at 4°C, and the pellets were resuspended into bacterial protein extraction B-PER reagent in phosphate buffer (Pierce, Rockford, IL) for total protein extraction. FanC-STa-BVDV-eltA2:B fusion protein has been purified according to [151], then the purified protein was examined by standard sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Statistic analysis:

Data were analyzed by student's t-test and used for comparison of the different treatments; the results were expressed as means ± standard deviations. P values were calculated to measure significances in differences, calculated P values of <0.001 was regarded as significant.

Table 3 Strains and plasmids used in subunit vaccine development

Strains or plasmids	Relevant properties	Reference
E. coli strains		
BL21	_E coli_ competent cell, _fhuA2 [lon] ompT gal [dcm] ΔhsdS_	Invitrogen
1297	_E. coli_ KI2, strain 711, _lac his trp proC phe_ Nal[r]	[1]
9196	FanC-STa-BVDV fusion cloned to pET28a, NheI/EagI in BL21 competent cell	This study
9210	E2 glycoprotein from BVDV type-1, NADL strain, Nde1/BamH1 cloned to pMAL-p5X in BL21 competent cell	This study
BVDV strain		
NADL	Flaviviridae, Pestivirus, Bovine viral diarrhea virus-1, NADL strain (ATCC VR-534).	ATCC
Plasmids		
pET28a	Cloning vector of pBR322 origin with BamHI-XhoI MCS, His-Tag, T7-Tag, T7-promoter, T7-transcription, T7-terminator, _lacI,_ Kan[r].	Invitrogen
pMAL-p5X	Cloning vector with MBP, encoded by the malE gene, protease Factor Xa used for cleavage of fusion protein from MBP, tac promoter (Ptac), IacI[q], AP[r].	New England Biolabs
p9196	FanC-STa-BVDV, NheI/EagI in pET28a	This study
p9210	BVDV- NADL E2 glycoprotein, Nde1/BamH1 in pMAL-p5X	This study
pK99	_E. coli_ KI2 strain 711 with 7.1-kb K99 BamHl fragment cloned into pBR322 generating plX12	[1]

Results

    Constructed fusion protein FanC-STa-E2 carried representative epitopes.

The whole *E. coli* K99 plasmid was received as a gift from Dr. R. E. Isaacson, at the

Veterinary and Biomedical Sciences Department, University of Minnesota, Minnesota,

USA (figure 2) and used to construct our strain in this study. The pK99 was analyzed and

shown to be composed of eight subunits, while the fanC is the major fimbriae. Mapping

of the major fanC fimbriae to detect the non conserved less antigenic exposed epitopes

(figure 3), which removed and replaced with $pSTa_{12}$ and BVDV epitopes. Those non

conserved fanC amino acid sequence 91-107, 116-126 and 154-62 has been removed for

the insertion of STa_{P12F} (NNTFYCCELCCN*F*ACAGC), the BVDV E2 B-cell epitope

(^{70}HTRALPTSVVF^{80}), and the E2 T-cell epitope (^{193}DQLLYKGGS^{201}), resulted in the

FanC-STa-E2 fusion protein (figure 4). Our final product is fanC-STa-E2 fusion protein.

Fusion FanC protein was transformed by electroporation into BL21 competent *E. coli* and

purified by the Ni-NTA purification system. The fusion protein was refolded,

concentration was mesuresed through Lawry protein assay and confirmed by Western

blotting and commasie blue staining before proceeding to the animal study (figure 5).

    The multiepitope FanC-STa-E2 fusion protein was safe and immunogenic.

Mice did not display any noticeable distress or adverse effects after i.p. immunized with

this 6xHis-tagged multiepitope FanC-STa-E2 fusion protein. Anti-K99, anti-STa and

anti-BVDV antibodies were developed. Adult mice were vaccinated with purified FanC

proteins which emulsified in Freund's complete adjuvant (FCA) in the primary injection

at day 1, followed by two booster injections with purified chimeric FanC proteins which

emulsified in Freund's incomplete adjuvant (FIA) on days 14 and 28 post primary

immunization. Both serum and fecal samples were collected weekly at day 0, 7, 13, 20, 27 and 32. The final sample collected 10 days later after the last immunization at day 38 and was used to test the antibody titers using ELISA. Bacterial adherence inhibition assay, neutralization activity of both STa enterotoxin and BVDV were evaluated. The titers were determined by the log10 value of the highest dilution with an OD >0.3.

The weekly collected sera were tested; they showed a detectable increase in the antibody titer along the experimental period with an increase in the serum IgG Anti-K99, anti-STa and anti-BVDV antibodies (figures 9, 10, 11). The booster immunization was able to potentiate and stimulate the mice immune response and increase the antibody titer in the three antigens. It was noticed that STa has the ability to evoke an immune responses which indicates that coupling of STa epitope to fanC fimbriae render STa more immunogenic.

The last sample, collected 10 days after the last immunization, was tested for antibody levels. Systemic IgG antibodies in serum samples and the secretory IgA mucosal antibodies from fecal samples supernatant were tested by ELISA. Anti-K99 IgG antibodies were detected in the serum of the immunized mice at titers (log10) of 3.611 ± 0.02595, while no anti-K99 IgG antibodies were detected in the serum sample from the control mouse (figure 12). Anti-K99 secretory IgA antibodies were detected (OD 405) of 0.2420 ± 0.04332 in the feces of mouse immunized with fanC chimeric protein. In contrast, no anti-K99 antibodies IgA were detected in the fecal sample from the control mouse (figure 15). Both anti-K99 IgG and IgA antibodies obtained from immunized mouse showed a significant difference comparing with the control (P < 0.001).

Anti-STa IgG antibodies in sera of immunized and control mice with 1:10 dilution were detected at (OD 405) of 0.2733 ± 0.05660 and 0.01438 ± 0.00496 respectively. The anti-STa IgG antibodies were significantly different from the control mouse ($P < 0.001$). Suggesting that STa epitope became immunogenic when expressed as a foreign antigen in chimeric FanC protein and could elicit anti-STa IgG antibodies (figure 13). There was no significant (ns) difference in the anti-STa IgA antibodies detected in the fecal samples of immunized mouse comparing with the control mouse (figure 16).

There was a significant difference in the anti-BVDV serum IgG antibody from immunized group comparing with the control, the titer (log10) was 3.479 ± 0.08771($P < 0.001$) (figure 14). Anti-BVDV IgA antibodies were detected at (OD 405) of 0.3287 ± 0.06879 in the fecal supernatant of mouse immunized with fanC chimeric protein ($P < 0.001$) (figure 17). Anti-BVDV IgA antibodies titer (log10) was measured in four immunizied mice (OD >0.3) and showed a significant difference with the control, 7263 ± 0.276 ($P < 0.05$) (figure 18). Anti-BVDV IgA was detected with a higher level comparing with anti-K99 IgA. Good immune response is expected to produce antibodies able to neutralize any invaders or its virulence factor. After the antibodies against our chimeric protein were inducted, the three antigens (K99, STa, and E2) were confirmed. Adherence and neutralization assay were tested to evaluate the biological functions of these antibodies in collected serum and fecal samples in vitro.

Antibodies in serum samples of the immunized mice significantly inhibited adherence of K99 fimbrial *E. coli* bacteria to INT-407 and IPEC-J2 cells. The bacterial adherence inhibition assay was carried out. It showed that the *E. coli* K99 able to bind to different types of K99. The IPEC-J1 and INT-407 were able to bind to K99

bacteria as they considered K99 receptor positive cell line [137]. The bacteria suspension

was incubated with the sera or fecal supernatants from mouse immunized with chimeric

fanC fimbriae (1:10) for 1 hour. Mixture was added to both $1X10^5$ IPEC-J1 and $5X10^5$

INT-407 seeded cells. After 1 hour of incubation, the plate was washed; the cells were

dislodged, resuspended, and plated on LB agar plates in serial dilutions. Colonies which

grown from LB agar were counted to calculate CFUs/ml. Serum anti-K99 antibodies

from immunized mouse was shown to inhibit the bacterial binding in cell culture in vitro

and reduced the adherence of K99 fimbrial ETEC bacteria to K99 receptor positive cell

lines. There was a significance difference between the immunized and control sera.

Bacteria adherence was significantly reduced more than ten folds. Binding of the bacteria

to INT-407, or IPEC-J1 cells was 11.33 ± 0.8819 $(x10^3)$ and 2.333 ± 0.6667 $(x10^3)$

respectively in immunized serum. The binding was significantly lower than the bacteria

incubated with the serum of the control, $(168.3 \pm 6.009$ $x10^3)$ and $(50.00 \pm 5.000$ $x10^3)$

respectively (P <0.001) (figure 19). Fecal antibodies showed no significant differences

between the immunized and the control, results were not confirmed after incubation with

fecal samples; this was due to the detachment of the cells when incubated with the fecal

sample supernatant.

Antibodies in serum samples of the immunized mice neutralized STa toxin. In

addition to their bacterial adherence blocking activity, the antibodies which produced by

the immunized mouse have the capability of neutralizing STa enterotoxin. STa is able to

increase the intracellular cyclic GMP level in host cells. In neutralization assays, the

purified 2ng STa toxin was incubated with antibodies from serum or fecal samples and

then used to treat T84 cells. The T84 cells were lysed with 0.1 M HCl and centrifuged.

The supernatants were collected to test the intracellular cyclic GMP concentration. A cyclic GMP EIA was carried out to measure the intracellular cGMP level in T84 cells, which indicates the toxicity of enterotoxins in those cells. Antibodies from immunized sera neutralize the STa toxin efficiently. This indicated by a significant reduction in the intracellular cGMP level. The cGMP level in the control and the immunized sera was 4.100 ± 0.7506 and 0.5425 ± 0.07157 pmol/ml respectively ($P < 0.001$) (figure 20). The fecal IgA antibodies did not show any significance difference between the immunized and the control groups.

Antibodies in serum samples of the immunized mice showed neutralizing activities against BVDV viral infection. BVDV neutralization assay was done to test the neutralizing ability of anti-BVDV antibodies utilizing the MDBK cells (ATCC CCL-22) and BVDV-NADL strain (ATCC VR-534). Briefly, serum samples were double fold serially diluted. The neutralization titer for the immunized serum was (≤ 640 dilutions) after three days of incubation with BVDV in MDBK cells. Neutralizing antibodies were detected in the immunized mice; they have the ability to block the cytpathic effect of NADL strain on MDBK cells *in vitro*. In our mice study, the anti-BVDV IgG antibody titer (log10) in the immunized group was significantly higher than the control 3.479 ± 0.08771 ($P < 0.001$). All serum samples in the control had no detectable neutralizing antibody (titers of 0) and a clear cytopathic effect was detected (figure 21).

In summary, we were not only effectively able to induce humoral immune responses by chimeric FanC proteins. But also obtained biologically functional antibodies that could block bacteria adherence to intestinal epithelial cells neutralize enterotoxins and BVDV in tissue culture *in vitro*.
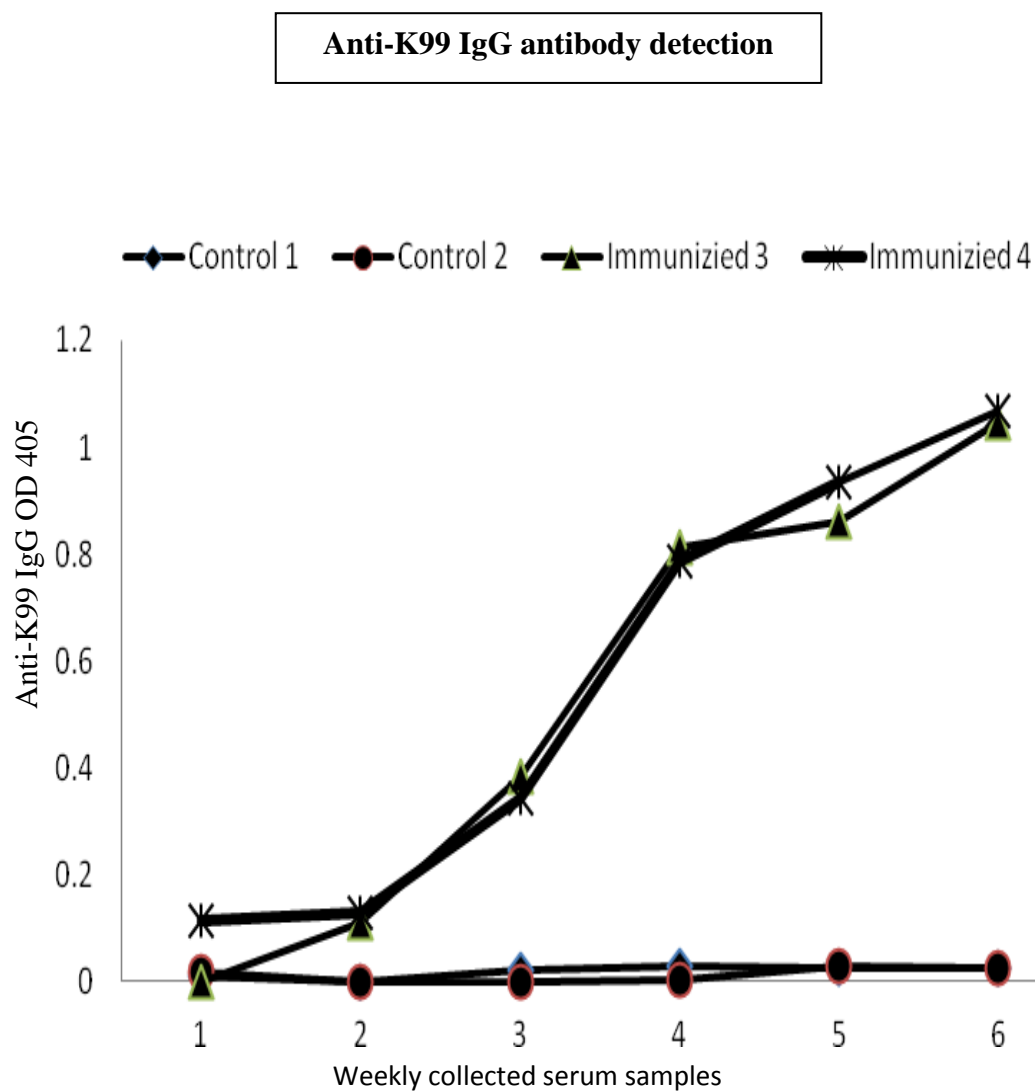
Fig.9. Anti-K99 IgG antibody detection. Serum samples from control and immunized mouse with chimeric fanC protein were tested along the experimental period (1-6 weeks). Each of the control and immunized mice was subdivided in two cages per group (control 1&2) and (immunized 3&4). Samples were been used as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgG (1:5000) was used as secondary antibody.
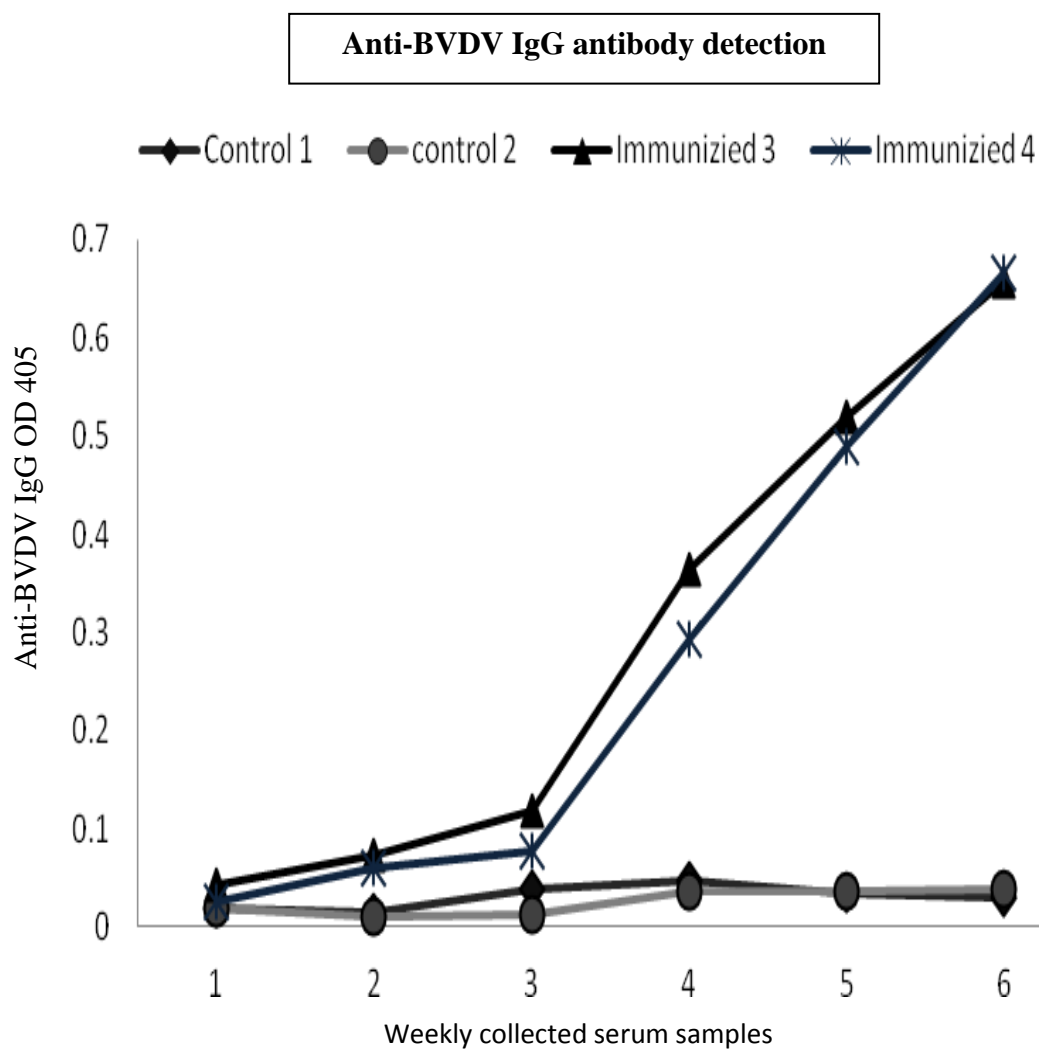
Fig.10. Anti-BVDV IgG antibody detection. Serum samples from control and immunized mouse with chimeric fanC protein were tested along the experimental period (1-6 weeks). Each of the control and immunized mice was subdivided in two cages per group (control 1&2) and (immunized 3&4). Samples were used as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgG (1:5000) was used as secondary antibody.
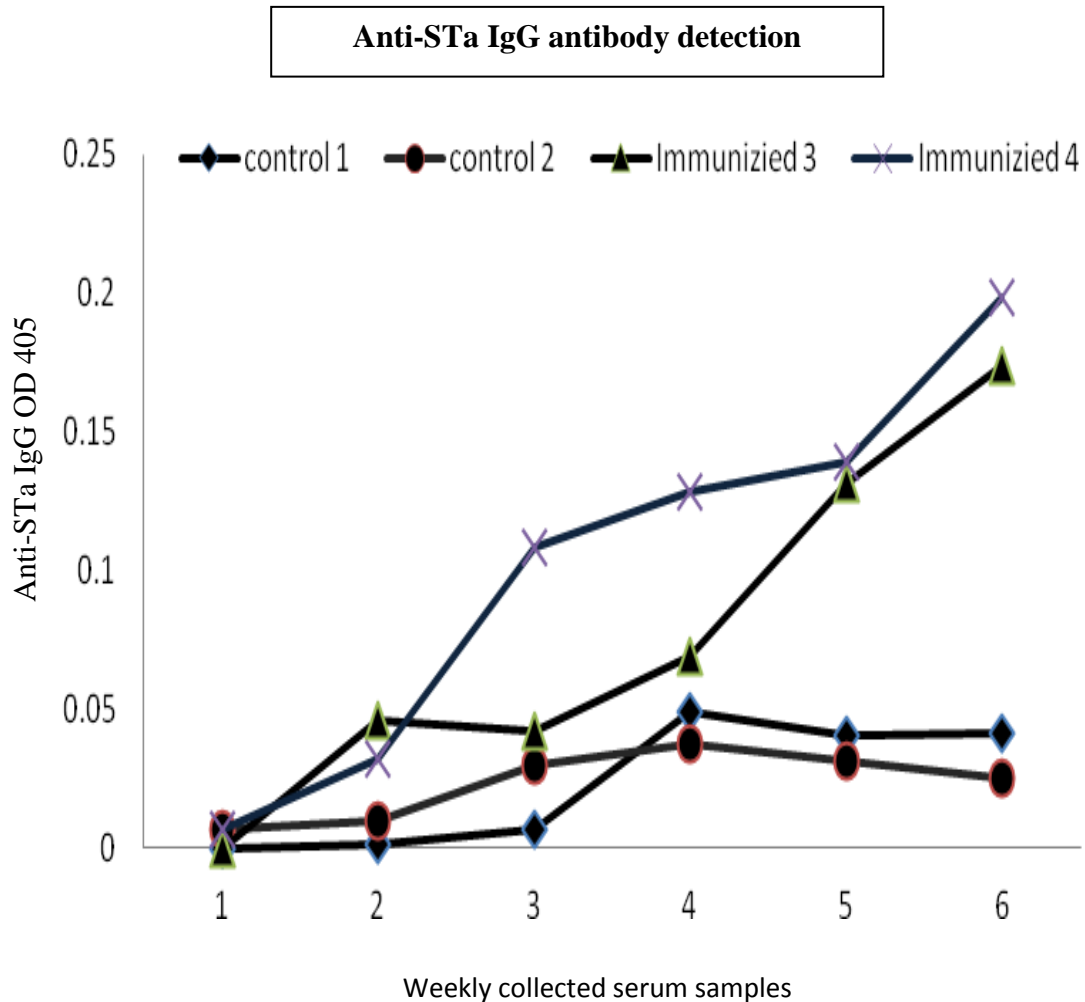
Fig.11. Anti-STa IgG antibody detection. Serum samples from control and immunized mouse with chimeric fanC protein were tested along the experimental period (1-6 weeks). Each of the control and immunized mice was subdivided in two cages per group (control 1&2) and (immunized 3&4). Samples were used as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgG (1:5000) was used as secondary antibody.
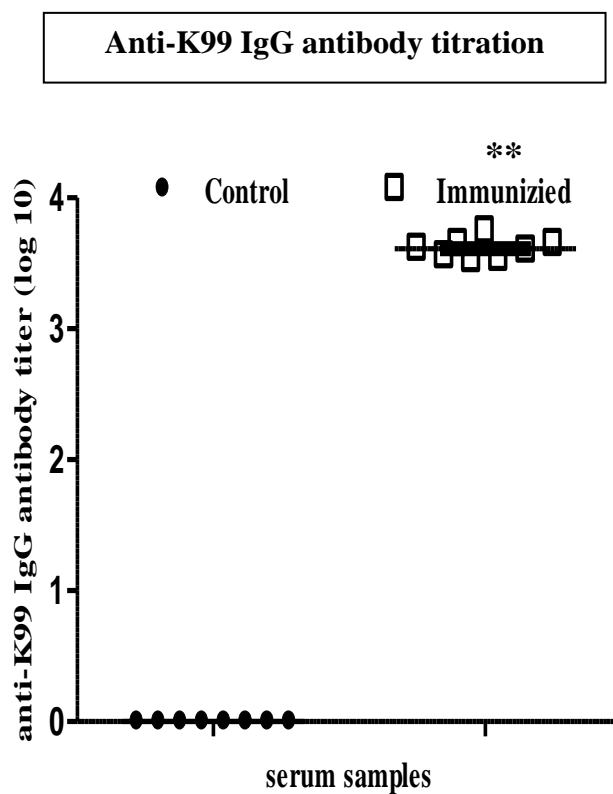
Fig.12. Anti-K99 IgG antibody titration ($\log_{10}$). Serum samples from control & immunized mice with chimeric fanC protein were used as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgG (1:5000) was used as secondary antibody. The error bars were calculated with the OD values >0.3, after subtraction of the background, of triplicate wells of samples.**,(P < 0.001).
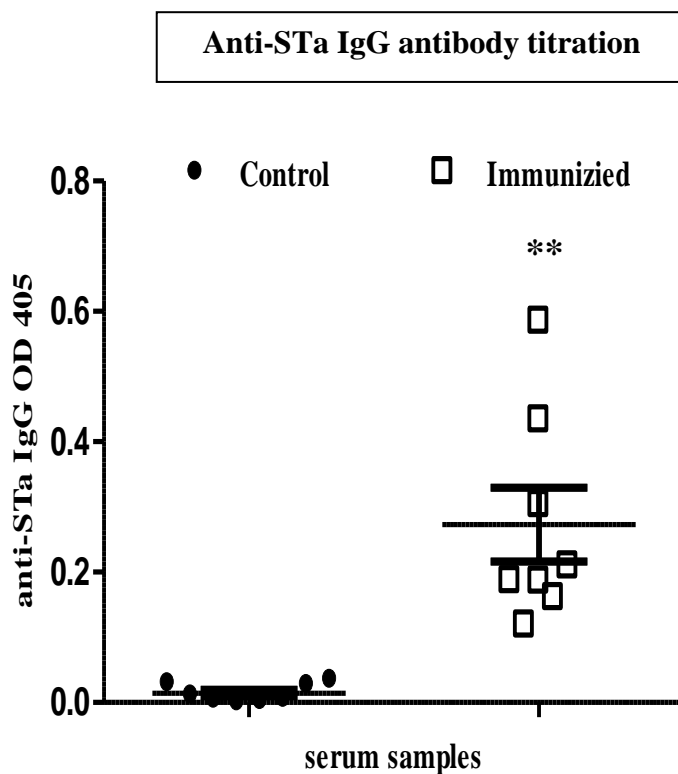
Fig.13. Anti-STa IgG antibody titration (OD 405). Serum samples from control & immunized mice with chimeric fanC protein were used as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgG (1:5000) was used as secondary antibody. Three samples showing OD >0.3, three with OD ≥0.2 and two showing an OD <0.2. Reading was taken after subtraction of the background in triplicate. The error bars indicate standard deviation. **,(P < 0.001).
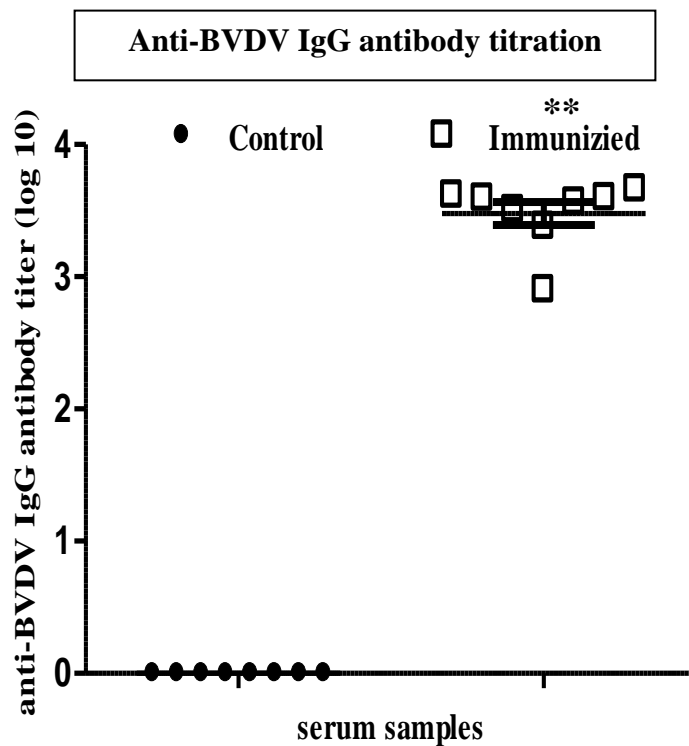
Fig.14. Anti-BVDV IgG antibody titration (log$_{10}$). Serum samples from control & immunized mice with chimeric fanC protein have were as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgG (1:5000) was used as secondary antibody. The error bars were calculated with the OD values >0.3, after subtraction of the background, of triplicate wells of samples.**,(P < 0.001).

Fig.15.Anti-K99 IgA antibody titration (OD405). Fecal samples supernatant, from control & immunized mice with chimeric fanC protein were used as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgA (1:5000) was used as secondary antibody. Two samples showing OD >0.3, two showing an OD >0.2 and remaining OD<.0.2 Reading was taken after subtraction of the background in triplicate. The error bars indicate standard deviation. **,(P < 0.001).

Anti-STa IgA antibody titration
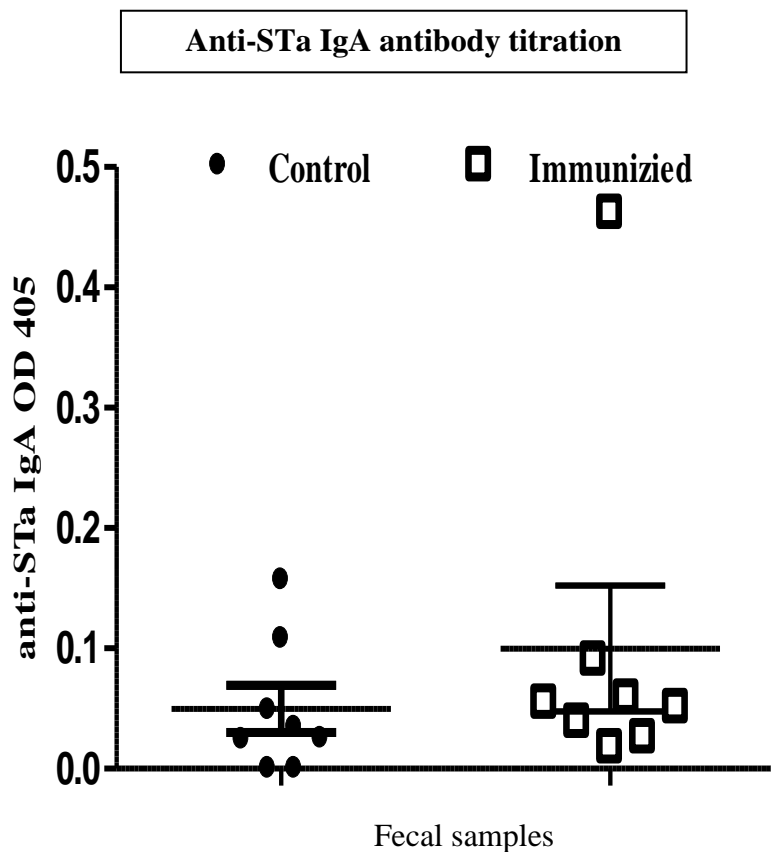
Fig.16. Anti-STa IgA antibody titration (OD405). Fecal samples supernatant, from control & immunized mice with chimeric fanC protein were used as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgA (1:5000) was used as secondary antibody. All samples showed no significant changes (ns). Reading was taken after subtraction of the background in triplicate. The error bars indicate standard deviation.
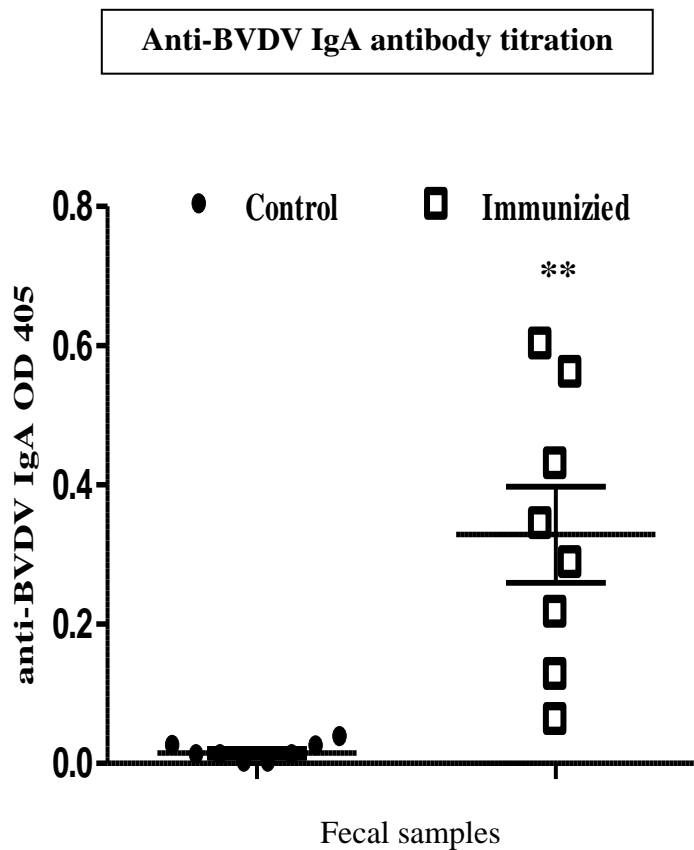
Fig.17. anti-BVDV IgA antibody titration (OD 405). Fecal samples supernatant, from control & immunized mice with chimeric fanC protein were used as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgA (1:5000) was used as secondary antibody. Four samples with OD >0.3, two with an OD >0.2 and two with OD<.0.2 Reading was taken after subtraction of the background in triplicate. The error bars indicate standard deviation. **,(P < 0.001).

Fig.18. Anti-BVDV IgA antibody titration ($\log_{10}$). Fecal samples from control & immunized mice with chimeric fanC protein have were as primary antibodies in ELISA while HRP-conjugated goat anti-mouse IgA (1:5000) was used as secondary antibody. The error bars were calculated with the OD values >0.3, after subtraction of the background, of triplicate wells of samples.**,(P < 0.05).

Bacterial adherence inhibition assay
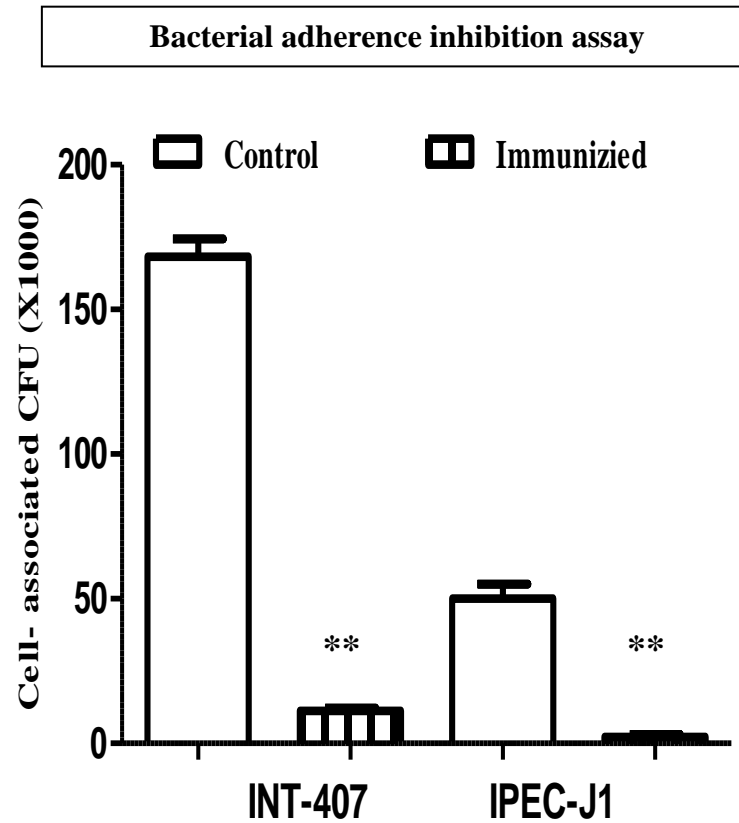
Fig.19. Bacterial adherence inhibition assay. Serum samples from control & immunized

mice with chimeric fanC protein, *Escherichia coli* K99 strain and two cell lines, IPEC-J1

and INT-407 were used. $1X10^5$ IPEC-J1 and $5X10^5$ INT-407 cells were seeded into each

well. The multiplicity of infection was set to 5 bacteria per cell. Samples incubated with

the bacteria then added to the cells. Cells were dislodged, serially diluted in PBS and

plated on LB agar plates overnight at 37°C. Colonies were counted and calculated as

CFU/ml (in thousthands). Error bars indicate standard deviations. **,($P < 0.001$).

Fig.20.Anti-STa antibody neutralization assay. Serum samples (1:10) from mouse vaccinated with purified chimeric FanC protein were used to neutralize 2ng STa. Mixture was added to T84 cells to detect the stimulation of intracellular cGMP level (pmole/ml) with a direct cGMP enzyme immunoassay kit (Assay Designs) to asecss the anti-STa antibody neutralization activity. Cell culture medium alone has been used as a negative control, serum samples from control and immunizied mouse were tested. Error bars indicate standard deviations. **,(P < 0.001).

Anti-BVDV antibody neutralization assay

Fig.21.Anti-BVDV antibody neutralization assay. (A) Negative control cell, (B) Positive control, (C) serum sample immunized group+ virus, (D) serum sample control group+virus. MDBK cells& cytopathic NADL strain in 0.1 TCID50. The serum neutralization titer $\leq$ 640 were able to neutralize the NADL virus in vitro. Arrows indicate cell clustering as CPE. No CPE was detected in the immunized group.

Discussion

The main objective of this project was the achievement of protection against diarrheal disease caused by *E. coli* K99 and BVDV. This was the first study reports the combination of one vaccine able to provide a protection against *E. coli* K99 and BVDV. Adhesins and enterotoxins induce fluid and electrolyte hypersecretion. ETEC control based on the blocking of the bacterial adherence to the host receptors and eliminating of enterotoxin activity in the host epithelial cells [36]. BVDV control includes the limitation of disease spread and/or reduction of the disease severity beside the prevention of the transplacental infection. The combination of T and B cell epitopes from E2 glycoprotein is a good approach for the protection with providing sterilizing immunity [123]. It is believed that the subunit vaccine candidates composed of an adhesin antigen, toxin antigen and BVDV epitopes or carrying a adhesin-toxin-BVDV fusion protein which could be a good stimulant for anti-adhesin, anti-toxin and anti-BVDV immunity which protect against both diseases.

Current vaccine strategy was to have one vaccine candidate that is able to provide a protection against *E. coli* K99 and BVDV infection. The subunit vaccine composed of a purified FanC fusion protein. Current immunization practice using purified pili or inactivated K99 fimbrial *E. coli* bacteria does not induce any antitoxin immunity to protect against enterotoxicity. As a small-size and poorly immunogenic molecule, STa alone does not stimulate anti-STa immunity. Therefore, even inactivated K99 fimbrial bacteria expressing STa toxin cannot stimulate immunity protecting against STa toxin. In contrast, since STa is potently toxic, any products carrying native STa are considered not safe. The secreted heat-stable enterotoxin a (STa) is the major secreted toxin in bovine

ETEC K99 infection which predispose to the secretory diarrhea [140]. Therefore, this makes STa toxoid an attractive target to be included in ETEC K99 vaccine. Data from this study indicate that the fusion antigen carrying the K99 antigen FanC and STa toxoid STa$_{P12F}$ elicited both anti-K99 and anti-STa antibodies. Moreover, antibodies in serum of the immunized mice exhibited activities to inhibit adherence of K99 fimbrial *E. coli* bacteria and to neutralize STa toxin.Genetic integration of three epitopes in the K99 fanC major fimbriae confirms the idea that it could be applied as a platform for developing effective multivalent vaccines against bovine diarrhea. Similarly, several studies were shown to target FanC major fimbrae of K99 in vaccine development. A subunit vaccine development from an edible transgenic soybean was used, immunize mice with extracted protein from the transgenic leaves which developed a high antibody titer and stimulate the mice immune system [84].

The idea behind utilizing chimeric fimbriae as a backbone for ETEC vaccine development with the insertion of several epitopes showed a great success in different studies. One study uses the *Escherchia coli* K88ac fimbriae to express LT & STa toxin epitopes, and used for rabbit immunization which stimulate the production of systemic IgG and mucosal IgA antibodies that neutralize Cholera toxin, STa toxin and prevent the K88ac *E. coli* adhesion [136]. Several in vitro studies were developed on cell culture to test the ability of the antibodies to prevent the bacterial adherence on the tissue culture and the antibody neutralization assay.

Measuring the antibody titer on a weekly collected serum samples, showing a gradually increase of the systemic IgG antibody along the experimental period for anti-K99, anti-STa and anti-BVDV antibodies (Fig.9, 10, 11), which indicate that the

chimeric fanC antigen was able to stimulate the immune response and the booster doses of antigen were able to evoke better immune response. Last collected serum and fecal samples ten days after the last immunization were tested. The antibody titer anti-K99, anti-STa and anti-BVDV antibodies IgG and IgA respectively were evaluated. It is noted that greater titers of anti-K99 and anti-BVDV E2 IgG antibodies were detected in the serum samples of the immunized mice. But anti-STa immune response was detected low in the immunized mice. Indeed, only two immunized mice had anti-STa IgG detected above the arbitrarily set-up OD cutoff point (0.3 after subtraction of background readings). The serum IgG antibody titer was much higher than the fecal IgA mucosal antibody titer along the experimental period. We relate the difference between the systemic and the mucosal antibody titer in our study due to the intraperitoneal (IP) route of antigen administration, which is a potent stimulant of the systemic immune system, but it is not good stimulant of mucosal antibody. Most of the commercial vaccines elicit systemic antibody due to the systemic homing specificity of the effector lymphocytes in the peripheral lymphnode [12].

Based on our results, the fusion antigen was able to significantly stimulate the production of anti-K99 IgG antibodies, which found to be anti-adhesin antibodies and significantly block the adherence of wild type K99$^+$ ETEC strain to a porcine intestinal epithelial cell line (IPEC-J1) and human caucasian embryonic intestine cell line (INT-407) in vitro. It was reported that porcine intestinal epithelial cell line (IPEC-J1) and human Caucasian embryonic intestine cell line (INT-407) have the ability to bind to ETEC-K99 bacteria in vitro [137]. Similarly, anti-adhesin antibodies against ETEC fimbriae have been developed and were able to block bacterial adhesion in vitro in tissue

culture. One study use E. coli K88ac fimbriae with embedded LT and STa epitopes and used for rabbit immunization, anti-K88ac, anti-LT and anti-STa antibodies has been developed which inhibit the adherence of K88ac fimbrial E. coli to porcine small intestinal enterocytes and neutralize cholera toxin and STa toxin [136]. Another study genetically fused nucleotides for tripartite FaeG-FedF-LT$_{(192)}$A2:B, and used for immunizations in mice and pigs, anti-K88, anti-F18, and anti-LT antibodies in immunized mice and pigs has been developed plus the ability of the anti-porcine antibodies elicited to neutralize cholera toxin and inhibited adherence against both K88 and F18 fimbriae [141].

Our antigen was able to induce anti-STa anti-toxin immunity. Our toxoid epitope STa$_{p12F}$ with a mutation in pro12 amino acid. It has been reported that the mutation of the pro12 and Ala13 of STa protein were significantly reduced the pSTa toxicity but retains the immunogenicity [128]. The stimulated anti-STa IgG antibodies were significantly increased between the immunized and the control group. The low anti-STa titers below our cutoff point 0.3 most likely resulted from the nature of low immunogenicity from STa or its derived toxoid antigens. Fusing additional copies of the STa toxoid (in a fusion antigen) was shown to facilitate overall anti-STa immunogenicity (data unpublished). Future studies to fuse two or more STa$_{P12F}$ copies into this FanC-STa-E2 fusion likely will induce greater titers of anti-STa antibodies. It was reported that STa became immunogenic when coupled to an appropriate large molecular weight carrier through chemical conjugation or recombinant fusion techniques and also it reduces the toxicity of STa molecule by mutagenesis to have a safe vaccine [67]. Several studies were developed to conjugate or genetically fuse STa to the carrier proteins to improve its

immunogensity [142]. One study showed that the porcine STa toxoids became immunogenic and produce neutralizing anti-STa antibodies after being genetically fused to a full-length porcine-type LT toxoid, LT_{R192G} [143].

The resulted anti-STa IgG antibody from this study have the ability to neutralize the toxicity of STa toxins efficiently in T84 human colonic adenocarcinoma cell line in vitro. There were no detected anti-STa IgA mucosal antibodies of the fecal samples supernatant. The development of anti-STa antibodies when integration of STa epitope in previous vaccine was reported. One study [136] embedded the STa toxoid epitope in the FaeG major subunit of *E. coli* K88ac fimbriae and used the chimeric protein for rabbit immunization, anti-STa antibodies were developed which also were able to neutralize the STa toxin. Another study reported an increase in the serum STa neutralizing antibodies when a recombinant native purified STa coupled to modified bovine serum albumin and used for immunization of female New Zealand white rabbits [144]. In summary, our chimeric antigen with STa toxoid epitope were able to elicit systemic anti-STa IgG neutralizing antibodies that could provide protection against STa toxicity *in vitro* in T84 cells but no mucosal anti-STa IgA antibodies were developed.

Realized the heterogeneity among BVDV isolates and existing challenges in developing broadly effective vaccines, we carried out this study to explore an alternative approach to develop vaccines against BVDV associated bovine diarrhea. Heterogeneity (genetically and more importantly antigenically) makes it nearly impossible for any live attenuated or inactivated vaccines derived from a single BVDV strain to provide broad protection. A cocktail of live attenuated strains could improve protective efficacy, but it increases the risk of virulence reversal of the product and also viral infections *in utero*.

For inactivated vaccines, mixing of multiple strains becomes less desirable due to difficulties in carrying sufficient antigens from individual strains. Subunit vaccines could overcome above disadvantages. Our primary target of including BVDV epitopes was to provide a protection against BVDV along with ETEC. Thus prevention of the transplacental transmission, PI infection and reduce the morbidity and mortality rate. This will increase the efficiency of animal production and health. In this study we did include one B-cell epitope and one T-cell epitope in our recombinant fanC protein to test the immunogensity of this protein in mice. B-cell epitopes are surface exposed regions of the protein, they are probably more mobile than interior regions, and so more hydrophilic and accessible to the immune system [145]. Epitopes were chosen from E2 glycoprotein because the majority of B and T-cell response is directed against E2 glycoprotein, plus the neutralizing effect of antibodies produced from E2 glycoprotein. The combination of B and T-cell epitopes is required to prevent of fetal infection [123]. Our results showed an increase in both, anti-BVDV IgG antibody and anti-BVDV IgA antibody titer. This increase in the systemic and mucosal antibodies resulted from including only two BVDV epitopes. The titer of anti-BVDV IgA antibody was much higher than the anti-K99 IgA which represents a promising tool for the control of BVDV infection.

Anti-BVDV IgG antibodies were able to neutralize the cpBVDV NADL strain in vitro in BVDV free MDBK cells. This means that antibodies have a protective neutralizing effect against the virus. It was reported that CPE is the earliest morphological change in response to cpBVDV infection in MDBK cells [146]. Our result showed that there was CPE in control group, while no CPE detected in the immunized group at serum dilution of ≤ 640. This indicates that the serum antibodies have the ability

to produce in vitro protection in MDBK cells. Clear cell morphology was detected under the microscope with immunized serum. The anti-BVDV IgG antibodies have the ability to reduce the severity of BVDV infection. Comparing with the control serum, more clustering of cells and clear cytoplasmic vacuolization were detected under the microscope. We cannot evaluate the mucosal anti-BVDV IgA neutralizing effect because the fecal sample supernatant cause death of MDBK cell line when incubated. Our neutralizing results was confirmed by the previous reshrachers, they stated that the glycoprotein E2 is the most immunogenic protein of BVDV and able to elicit high titers of strong neutralizing antibodies after infection and/or vaccination [108, 110, 139]. Several studies showing similar results of neutralizing antibodies when targeting E2 glycoprotein in BVDV vaccine, the only difference is that we were able to include only two BVDV epitopes not the whole E2 glycoprotein and get neutralizing antibodies. One experimental study in sheep tested two conventionally inactivated BVDV vaccine and one multivalent E2 subunit vaccine, all vaccines induced neutralizing antibodies against all challenge strains [147]. Another study showed that the recombinant BVDV-E2 protein, through a recombinant baculovirus (Ac-E2pol), neutralizing immune response against BVDV NADL strain were developed in the vaccinated mice after three doses of the antigen [148]. One more study used the recombinant vesicular stomatitis virus (VSV-E2) encoding BVDV E2 glycoprotein to induced the a recombinant E2 protein, BALB:c, the mice developed a specific BVDV neutralizing response after intranasal vaccine inoculation which lasted for at least 180 days post inoculation [149]. The BVDV NADL E2 glycoprotein neutralizing antibodies might have a cross protection with other BVDV strains which give a brooder protection against BVDV infection. One study showed that

the BVDV E2 glycoprotein of NADL strain was used to construct vaccinia virus recombinants expressing E2 to immunize mice of three H-2 haplotypes, serum samples from mice showed a high levels of neutralizing antibodies to NADL strain and were able to neutralize the Oregon C24V reference strain but failed to neutralize the Osloss reference [150].

Due to the high cost of subunit vaccine preparation, we tried to construct a live vaccine protect against ETEC and BVDV. Advantage of this technology, stimulation of strong humoral and cell-mediated immune responses that can be highly effective in preventing or clearing the later infection with low coast. A fusion antigen of recombinant fusion protein composed of (FanC-pSTa-BVDV E2) which is fused to LtA2-LtB has been constructed. Two constructs with different promoters has been used to compare the protein expression and use the one with high expression level. Our results showed that the construct was not completely assembled to holotoxin in both constructs as confirmed by western blotting. We were not able to detect our epitopes attached to LtA2 subunit, only the pentamer B subunit was verified by Western blotting. My future recommendation, retain partial of the LTA1 peptide (of C-terminus) or constructing of full length toxin like structure with mutation in A subunit to decrease its enzymatic activity. This will stable the holotoxin structure and elaborate potent immune response.

In summary, the FanC-pSTa-BVDV E2 fusion antigen was constructed by replacing non conserved exposed epitopes on FanC with STa and BVDV B and T-cell epitopes. It should be pointed out that this study examined mainly the immunogenicity of the constructed FanC-STa-E2 fusion antigen. Although data showed that this fusion antigen elicited specific antibodies in mice and the antibodies showed neutralizing

activity against ETEC and BVDV viral infection, its candidacy as a subunit vaccine against ETEC neonatal diarrhea in calves and BVDV diarrhea at other ages can only be determined in large-samples and well-designed field trials. Future studies will be also needed for the development of a broad-spectrum vaccine against bovine diarrhea.

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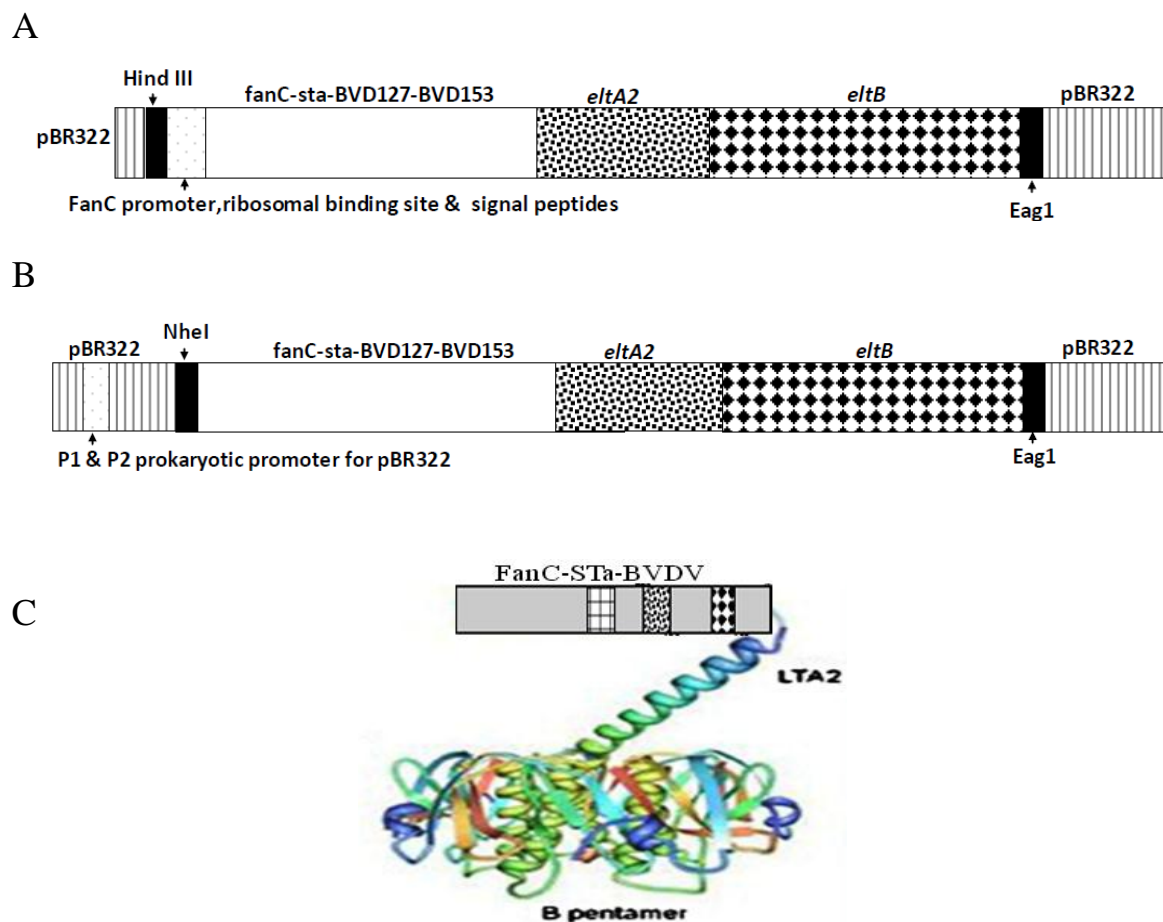
Supplementary Data

Primer	Sequence (5`- 3`)

FanC-ltA2-Nhe1-F

5`- CATATGGCT AGCGATGAAAAAAACACTCCTAGCTATTATCTTA-3

FanC- LtA2- HindIII-F

5`-ATCATCGATAAGCTTTAGGGAATGGCTATGTTTTCTGGT-3

LtB-EagI-R   5`GATCGGTATTGCGGCCGCTACCGC-3`

FanC-LtA2-F2

5`-GCATCCTTCTTAGTCACTTATATGCATCATGCACCACAAGGTTGTGG-3`

FanC-LtA2-R2

5`-CCACAACCTTGTGGTGCATGATGCATATAAGTGACTAAGAAGGATGC-3`

Supplement- Table-1:

Primers used to construct FanC-STa-BVDV-LtA2-LtB in pBR322, underlined are the restriction enzyme site. The first construct was used for the construction of fanC chimeric gene in pBR322 with NheI/EagI restriction enzyme. The second construct was the chimeric fanC- LtA2-LtB gene in pBR322 with HindIII /EagI restriction enzyme site. FanC-LtA2-F2& FanC-LtA2-R2 are two overlapping primers that connect the chimeric fanC gene with LtB subunit. While LtB-EagI-R is the reverse primer used in both constructs.

Supplement-Table-2: Strains and plasmids used in this research project

Strain or plasmid	Relevant properties	Reference
***E. coli* strains**		
BL21	*E coli* competent cell, *fhuA2 [lon] ompT gal [dcm] ΔhsdS*	Invitrogen
9196	FanC-STa-BVDV fusion cloned to pET28a, NheI/EagI in BL21 competent cell	This study
9205	FanC-STa-BVDV-LtA2-LtB, NheI/EagI in pBR322 in DH5α	This study
9300	FanC-STa-BVDV-LtA2-LtB, HinIII/EagI in pBR322 in DH5α	This study
Plasmids		
p9196	FanC-STa-BVDV, NheI/EagI in pET28a	This study
p9205	FanC-STa-BVDV-LtA2-LtB, NheI/EagI in pBR322	This study
p9300	FanC-STa-BVDV-LtA2-LtB, HinIII/EagI in pBR322	This study
p9073	FedF-LTA2-LTB, NheI/EagI in pBR322	Our lab

A

Hind III

pBR322   fanC-sta-BVD127-BVD153   eltA2   eltB   pBR322

FanC promoter,ribosomal binding site & signal peptides

Eag1

B

pBR322   NheI   fanC-sta-BVD127-BVD153   eltA2   eltB   pBR322

P1 & P2 prokaryotic promoter for pBR322

Eag1

C

FanC-STa-BVDV

LTA2

B pentamer

Supplement- figure-1:

(A). Stock# p9300 the fanC-STa-BVD127-BVD153/LtA2-LtB in pBR322 HindII/ EagI @ DH5-α with FanC signal peptide, FanC promoter and FanC ribosomal binding site. (B) Stock# p9205, fanC-STa-BVD127-BVD153/LtA2-LtB in pBR322 NheI/EagI @ DH5-α, Include pBR322 tetracycline gene & FanC signal peptide, and removed FanC promoter and FanC ribosomal binding site. (C). three dimension diagram of our expected fake holotoxin like structure in both constructs 9205 & 9300.

A
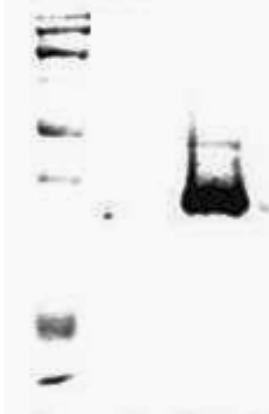
(A) Anti-CT (1:3000) Western blotting, 12% PAGE gels, detect the LTB pentamer subunit from un boiled protein, samples of strain 9300& 9205.

B   C   D

Supplement- figure-2:

Detection fusion protein fanC-STa-BVDV-LTA2-LTB in pBR322. (B) Western blotting, anti-K99 polyclonal antibodies 1:1000. (C) Western blotting, anti-STa monoclonal antibodies 1:2500. (D) Western blotting, anti-BVDV NADL strain polyclonal antibodies 1:1000. No band was detected which means that the epitopes were not recognized and the protein is not completely assembled to the holotoxin structure.

ATG GGC AGC AGC CAT CNT CAT NNT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC CAT ATG **GCT AGC** ACA CTC CTA GCT ATT ATC TTA GGT GGT ATG GCT TTT GCG ACT ACC AAT GCT TCT GCG AAT ACA GGT ACT ATT AAC TTC AAT GGC AAA ATA ACG AGT GCT ACT TGT ACA ATT GAG CCT GAG GTC AAT GGT AAT CGT ACA TCA ACT ATA GAT CTT GGG CAG GCT GCT ATT AGT GGT CAT GGC ACT GTA GTG GAT TTT AAA CTA AAA CCA GCG CCC GGC AGT AAT GAC TGC CTA GCG AAA ACA AAT GCT CGT ATT GAC TGG TCT <u>AAC AAC ACA TTT TAC TGC TGT GAA CTT TGT TGT AAT TTT GCC TGT GCT GGA TGT</u> GGA AAT ACT GCT GCT AAA GGA TAC CAT ATG ACT TTG <u>CAT ACA AGA GCC TTG CCG ACC AGT GTG GTA TTC</u> GCT AAT ATT AAT ACT TCA TTC ACT ACG GCT GAA TAC ACT CAC ACT TCT GCA ATT CAG TCA TTT AAC TAT TCA GCC CAG CTG <u>GAC CAA CTA CTA TAC AAA GGG GGC TCT</u> GGT GGA TAT AAA GCT GGC GTA TTT ACT ACT TCA GCA TCC TTC TTA GTC ACT TAT ATG TAA TAT TTA AAG TAT TTT ACA TTG **CGG CCG** CAC TCG AGC ACC ACC ACC ACC ACC ACT GAG ATC CGG CTG CTA ACA AAG CCC GAA AGG AAG CTG

Supplement- DNA sequence-1:

FanC-STa-E2 DNA sequence. Underlined are the three inserted epitopes pSTa$_{12}$, BVDV E2 B cell and BVDV E2 T cell respectively. Bold gray background is the restriction sites NheI & EagI.

ATG TCC GCT TTC TGG TAT GCC GTG CGT ACT GCG GTG ATC AAC GCC
GCC AGC GGT CGT CAG ACT GTC GAT GAA GCC CTG AAA GAC GCG CAG
ACT AAT TCG AGC TCG AAC AAC AAC AAC AAT AAC AAT AAC AAC AAC
CTC GGG ATC GAG GGA AGG ATT TCA CAT ATG CAC TTG GAT TGC AAA
CCT GAA TTC TCG TAT GCC ATA GCA AAG GAC GAA AGA ATT GGT CAA
CTG GGG GCT GAA GGC CTT ACC ACC ACT TGG AAG GAA TAC TCA CCT
GGA ATG AAG CTG GAA GAC ACA ATG GTC ATT GCT TGG TGC GAA GAT
GGG AAG TTA ATG TAC CTC CAA AGA TGC ACG AGA GAA ACC AGA TAT
CTC GCA ATC TTG **CAT ACA AGA GCC TTG CCG ACC AGT GTG GTA TTC**
AAA AAA CTC TTT GAT GGG CGA AAG CAA GAG GAT GTA GTC GAA ATG
AAC GAC AAC TTT GAA TTT GGA CTC TGC CCA TGT GAT GCC AAA CCC
ATA GTA AGA GGG AAG TTC AAT ACA ACG CTG CTG AAC GGA CCG GCC
TTC CAG ATG GTA TGC CCC ATA GGA TGG ACA GGG ACT GTA AGC TGT
ACG TCA TTC AAT ATG GAC ACC TTA GCC ACA ACT GTG GTA CGG ACA
TAT AGA AGG TCT AAA CCA TTC CCT CAT AGG CAA GGC TGT ATC ACC
CAA AAG AAT CTG GGG GAG GAT CTC CAT AAC TGC ATC CTT GGA GGA
AAT TGG ACT TGT GTG CCT GGA **GAC CAA CTA CTA TAC AAA GGG GGC**
TCT ATT GAA TCT TGC AAG TGG TGT GGC TAT CAA TTT AAA GAG AGT
GAG GGA CTA CCA CAC TAC CCC ATT GGC AAG TGT AAA TTG GAG AAC
GAG ACT GGT TAC AGG CTA GTA GAC AGT ACC TCT TGC AAT AGA GAA
GGT GTG GCC ATA GTA CCA CAA GGG ACA TTA AAG TGC AAG ATA GGA
AAA ACA ACT GTA CAG GTC ATA GCT ATG GAT ACC AAA CTC GGA CCT
ATG CCT GGA TCC GAA TTC CCT GCA GGT AAT TAA

Supplement- DNA sequence-2:

BVDV-E2 DNA sequence. Underlined are the BVDV E2 B cell and BVDV E2 T cell

respectively. Bold gray background is the restriction sites NdeI & BamHI.

ATG AAA TCT AAC AAT GCG CTC ATC GTC ATC CTC GGC ACC GTC ACC
CTG GAT GCT GTA GGC ATA GGC TTG GTT ATG CCG GTA CTG CCG GGC
CTC TTG CGG GAT ATC GTC CAT TCC GAC AGC ATC GCC AGT CAC TAT
GGC GTG CT**G CTA GC**G **ATG** AAA AAA ACA CTC CTA GCT ATT ATC TTA
GGT GGT ATG GCT TTT GCG ACT ACC AAT GCT TCT GCG AAT ACA GGT
ACT ATT AAC TTC AAT GGC AAA ATA ACG AGT GCT ACT TGT ACA ATT
GAG CCT GAG GTC AAT GGT AAT CGT ACA TCA ACT ATA GAT CTT GGG
CAG GCT GCT ATT AGT GGT CAT GGC ACT GTA GTG GAT TTT AAA CTA
AAA CCA GCG CCC GGC AGT AAT GAC TGC CTA GCG AAA ACA AAT GCT
CGT ATT GAC TGG TCT <u>AAC AAC ACA TTT TAC TGC TGT GAA CTT TGT TGT</u>
<u>AAT TTT GCC TGT GCT GGA TGT</u> GGA AAT ACT GCT GCT AAA GGA TAC
CAT ATG ACT TTG <u>CAT ACA AGA GCC TTG CCG ACC AGT GTG GTA TTC</u>
GCT AAT ATT AAT ACT TCA TTC ACT ACG GCT GAA TAC ACT CAC ACT TCT
GCA ATT CAG TCA TTT AAC TAT TCA GCC CAG CTG <u>GAC CAA CTA CTA</u>
<u>TAC AAA GGG GGC TCT</u> GGT GGA TAT AAA GCT GGC GTA TTT ACT ACT
TCA GCA TCC TTC TTA GTC ACT TAT ATG C**A**T CAT GCA CCA CAA GGT
TGT GGA AAT TCA TCA AGA ACA ATC ACA GGT GAT ACT TGT AAT GAG
GAG ACC CAG AAT CTG AGC ACA ATA TAT CTC AGG GAA TAT CAA TCA
AAA GTT AAG AGG CAG ATA TTT TCA GAC TAT CAG TCA GAG GTT GAC
ATA TAT AAC AGA ATT CGG GAT GAA TTA TGA ATG AAT AAA GTA AAA
TGT TAT GTT TTA TTT ACG GCG TTA CTA TCC TCT CTA TAT GCA CAC GGA
GCT CCC CAG ACT ATT ACA GAA CTA TGT TCG GAA TAT CGC AAC ACA
CAA ATA TAT ACG ATA AAT GAC AAG ATA CTA TCA TAT ACG GAA TCG
ATG GCA GGC AAA AGA GAA ATG GTT ATC ATT ACA TTT AAG AGC GGC
GAA ACA TTT CAG GTC GAA GTC CCG GGC AGT CAA CAT ATA GAC TCC
CAG AAA AAA GCC ATT GAA AGG ATG AAG GAC ACA TTA AGA ATC ACA
TAT CTG ACC GAG ACC AAA ATT GAT AAA TTA TGT GTA TGG AAT AAT
AAA ACC CCC AAT TCA ATT GCG GCA ATC AGT ATG GAA AAC TAG TAG

CGG CCG ACG CGC TGG GCT ACG TCT TGC TGG CGT TCG CGA CGC GAG GCT GGA TGG CCT TCC CCA TTA TGA TTC TTC

Supplement- DNA sequence-3:

Stock 9205 holotoxin like structure. FanC-STa-E2-LtA2-LtB includes fanC signal peptide (green). Underlined are the three inserted epitopes pSTa$_{12}$, BVDV E2 B cell and BVDV E2 T cell respectively. Bold gray background is the restriction sites NheI & EagI. Blue is the LtA2 sequence, red is the LtB sequence.

T TCT CAT GTT TGA CAG CTT ATC ATC GAT **AAG CTT** TAG GGA ATG GCT
ATG TTT TC**T GGT GAT T**CC ACG GAA CTA AAA AA**T AAT ATC** GAA CAA
TGG AGA ATC TAG **ATG** AAA AAA ACA CTG CTA GCT ATT ATC TTA GGT
GGT ATG GCT TTT GCG ACT ACC AAT GCT TCT GCG AAT ACA GGT ACT
ATT AAC TTC AAT GGC AAA ATA ACG AGT GCT ACT TGT ACA ATT GAG
CCT GAG GTC AAT GGT AAT CGT ACA TCA ACT ATA GAT CTT GGG CAG
GCT GCT ATT AGT GGT CAT GGC ACT GTA GTG GAT TTT AAA CTA AAA
CCA GCG CCC GGC AGT AAT GAC TGC CTA GCG AAA ACA AAT GCT CGT
ATT GAC TGG TCT <u>AAC AAC ACA TTT TAC TGC TGT GAA CTT TGT TGT AAT</u>
<u>TTT GCC TGT GCT GGA TGT</u> GGA AAT ACT GCT GCT AAA GGA TAC CAT
ATG ACT TTG <u>CAT ACA AGA GCC TTG CCG ACC AGT GTG GTA TTC</u> GCT
AAT ATT AAT ACT TCA TTC ACT ACG GCT GAA TAC ACT CAC ACT TCT
GCA ATT CAG TCA TTT AAC TAT TCA GCC CAG CTG <u>GAC CAA CTA CTA</u>
<u>TAC AAA GGG GGC TCT</u> GGT GGA TAT AAA GCT GGC GTA TTT ACT ACT
TCA GCA TCC TTC TTA GTC ACT TAT ATG CA T CAT GCA CCA CAA GGT TGT
GGA AAT TCA TCA AGA ACA ATC ACA GGT GAT ACT TGT AAT GAG GAG
ACC CAG AAT CTG AGC ACA ATA TAT CTC AGG GAA TAT CAA TCA AAA
GTT AAG AGG CAG ATA TTT TCA GAC TAT CAG TCA GAG GTT GAC ATA
TAT AAC AGA ATT CGG GAT GAA TTA TGA ATG AAT AAA GTA AAA TGT
TAT GTT TTA TTT ACG GCG TTA CTA TCC TCT CTA TAT GCA CAC GGA GCT
CCC CAG ACT ATT ACA GAA CTA TGT TCG GAA TAT CGC AAC ACA CAA
ATA TAT ACG ATA AAT GAC AAG ATA CTA TCA TAT ACG GAA TCG ATG
GCA GGC AAA AGA GAA ATG GTT ATC ATT ACA TTT AAG AGC GGC GAA
ACA TTT CAG GTC GAA GTC CCG GGC AGT CAA CAT ATA GAC TCC CAG
AAA AAA GCC ATT GAA AGG ATG AAG GAC ACA TTA AGA ATC ACA TAT
CTG ACC GAG ACC AAA ATT GAT AAA TTA TGT GTA TGG AAT AAT AAA
ACC CCC AAT TCA ATT GCG GCA ATC AGT ATG GAA AAC TAG TAG**CGG**

CCG ACG CGC TGG GCT ACG TCT TGC TGG CGT TCG CGA CGC GAG GCT GGA TGG CCT TCC CCA TTA TGA TTC TTC

Supplement- DNA sequence-4:

Stock 9300 holotoxin like structure. FanC-STa-E2-LtA2-LtB includes FanC promoter, ribosomal binding site and signal peptide (green). Underlined are the three inserted epitopes pSTa$_{12}$, BVDV E2 B cell and BVDV E2 T cell respectively. Bold gray background is the restriction sites HindIII & EagI. Blue is the LtA2 sequence, red is the LtB sequence.