Efficient production of human norovirus-specific IgY in egg yolks by vaccination of hens with a recombinant vesicular stomatitis virus expressing VP1 protein

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Yang Zhu, B.S.

Graduate Program in Food Science and Technology

The Ohio State University

2014

Thesis Committee:

Dr. Jianrong Li, Advisor

Dr. Melvin Pascall

Dr. Xin Li

Abstract

Human norovirus (NoV) is responsible for more than 95% of outbreaks of acute non-bacterial gastroenteritis worldwide and over 50% of foodborne illnesses in the US. Despite significant health, social, and economical burden it causes, no vaccine or antiviral drug available for this virus. This is due in major part to the fact that human NoV cannot be grown in cell culture and lacks a small animal model for pathogenesis study. Recent epidemiological studies showed that severe clinical outcomes including death are often associated with high-risk populations such as infants, children, the elderly, and immunocompromised individuals. There is an urgent need to develop an effective therapeutic agent for human NoV.

Chicken immunoglobulin Y (IgY)-based passive immunization has been shown to be an effective strategy to prevent and treat many enteric viral diseases such as rotavirus. In the present study, we developed an efficient approach to generate a high titer of human

NoV-specific IgY in chicken yolks using recombinant vesicular stomatitis virus (rVSV-VP1) expressing human NoV capsid protein (VP1) as an antigen. We first demonstrated that rVSV-VP1 replicated efficiently in a chicken cell line and VP1 protein can be highly expressed by VSV vector. Subsequently, White Leghorn chickens were immunized with recombinant rVSV-VP1 by intramuscular route or combination of intramuscular and nasal drop route. After immunization, eggs were collected daily and IgYs were purified from each egg. It was found that the purified IgY strongly reacted with human NoV virus-like particles (VLPs) by both enzyme linked immunosorbant assay (ELISA) and Western blot. In addition, hens vaccinated by intramuscular route triggered significantly higher human NoV-specific IgY than by the combination of intramuscular and nasal drop route. At week 4 post-vaccination, human NoV-specific IgY reached 4.8 mg per yolk in intramuscular vaccinated group, which was approximately 3 times more than that in the combined vaccination group. .

Next, we determined whether human NoV-specific IgY has potential antiviral activity. Since human NoV is uncultivable, there is no standard virus-antibody neutralizing assay available. However, it is known that human NoV utilizes histo-blood

group antigens (HBGAs) as functional receptors for attachment and subsequent viral entry. Importantly, we found that the purified human NoV-specific IgY efficiently blocked the binding of human NoV VLPs to all three types (A, B and O) of HBGA receptors using a saliva-based HBGA blocking assay. This result suggests that human NoV-specific IgY may have antiviral activity since blockage of viral receptor binding will likely inhibit viral attachment and entry.

In order to utilize human NoV-specific IgYs as immunological supplements in food products, the thermal and pH stability of IgY was investigated. It was found that the receptor blocking activity of IgY remained stable at temperature below 70 ^{o}C and at pH ranging from 4 to 9.

In summary, we developed a highly efficient bioreactor to produce human NoV-specific IgY from egg yolks by intramuscular vaccination of hens with a live attenuated rVSV-VP1 as an antigen. The human NoV-specific IgY was highly capable of blocking the interaction between human NoV receptors and VLPs. Thus, chicken IgY could be a practical strategy for large-scale production of anti-human NoV antibodies for potential

use in passive immunization against human NoV infection, as well as for therapeutic and diagnostic purposes.

Acknowledgments

I would like to thank all the people who have mentored me make this thesis possible.

Foremost, I would like to express my sincere gratitude to my advisor Dr. Jianrong Li for his patience, motivation, efforts, and the continuous support during my Master study at OSU. His immense knowledge, inspiration, and guidance helped me in all the time of my research and thesis writing. Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Melvin Pascall and Dr. Xin Li, for their time, commitment, and insightful suggestions.

I also want to thank Dr. Dr. Michael Lilburn for providing chickens for animal study. In addition, I want to thank the past and present members of Dr. Jianrong Li's group: Brie Coyle, Dr. Yu Zhang, Dr. Yuanmei Ma, Dr. Hui Cai, Elbashir Araud, Jing

Sun, Erin DiCaprio, Fangfei Lou, Yue Duan, Rongzhang Wang, and Xueya Liang for our stimulating discussions, for the days and nights we were working together, and also for all the fun we have had in the last two years.

Last but not the least, I wish to thank my family and friends for being a constant source of support – emotional, moral –no matter where I am and this thesis would certainly not have existed without them.

Vita

June 2008 .....Wenling High School, China

2012.....B.E. Food Science and Engineering,
Northwest A&F University

2012 to present .....Student Clerical Assistant,
Food Science and Technology,
The Ohio State University

Publications

Yuanmei Ma, Yongwei Wei, Xiaodong Zhang, Yu Zhang, Hui Cai, Yang Zhu, Konstantin Shilo, Michael Oglesbee, Steven Krakowka, Sean Whelan, and Jianrong Li. 2013. Messenger RNA cap methylation influences pathogenesis of vesicular stomatitis virus in vivo. Journal of Virology.

Fields of Study

Major Field: Food Science and Technology

Table of Contents

List of Tables

List of Figures

CHAPTER 1

CHAPTER 2

Abbreviations

CDC – Centers for Disease Control and Prevention

CPE – Cytopathic effect

DMEM – Dulbecco's modified eagle medium

ELISA – Enzyme-linked immunosorbant assay

EM – Electron Microscopy

FBS – Fetal bovine serum

FCV – Feline calicivirus

EDTA – Ethylenediamine tetraacetic acid

FDA – Food and Drug Administration

GI – Gastrointestinal

HAV – Hepatitis A virus

HBGA – Histo-blood group antigen

HuNoV – Human norovirus

MEM – Minimal essential medium

MOI – Multiplicity of infection

NIAID – National institute of Allergy and Infectious Diseases

NoV – Norovirus

ORF – Open reading frame

PBS – Phosphate buffered saline

PFU – Plaque forming units

RdRp – RNA-dependent RNA polymerase

RNA – Ribonucleic acid

SDS-PAGE – Sodium dodecyl sulfate- polyacrylamide gel electrophoresis

TEM- Transmission electron microscopy

Vero – An african green monkey kidney cell line

VLP – Virus-like particle

VSV – Vescicular Stomatitis virus

CHAPTER 1

LITERATURE REVIEW

1.1. Foodborne virus

Foodborne illnesses are defined as infections or irritations of the gastrointestinal (GI) tract caused by ingestion of contaminated food/beverages containing harmful bacteria, parasites, viruses, or chemicals (WHO). Human illness from the consumption of contaminated foods was recognized long before the understanding of the role of pathogens in foodborne disease. In recent years, foodborne illnesses are becoming the number one food safety concern among the most developed and developing countries due to frequent international travel and global food trade（63）. Among the microbes (viruses, bacteria, fungi, parasites, toxins, or prions) that can cause foodborne disease, viruses are the major cause of foodborne gastroenteritis in the United States and most other countries.

Mead et al. published a comprehensive statistical analysis of illnesses, hospitalizations, and deaths due to foodborne diseases in the United States in 1999. At that time, there were approximately 76 million illnesses, 323,914 hospitalizations, and

5,194 deaths each year in the United States alone. Notably, viruses, bacteria, and parasites accounted for 67%, 30%, and 2.6% of illnesses attributable to foodborne transmission (78). Recently, the public health officials at the Centers for Disease Control and Prevention (CDC) claim that the annual cases of foodborne illnesses in the United States has decreased to 48 million, or about 17% of the population get sick each year, leading to approximately 128,000 hospitalizations and 3,000 fatalities, and more than 60% of these illness caused by viruses(57).

According to WHO (1997), the cost for medical charges and lost productivity in the United States due to the foodborne disease caused by the major food-associated pathogens alone is up to 35 billion dollars annually. Thus, food contamination also poses an enormous social and economic burden on communities, in addition to its threat to human health.

CDC (2010) demonstrated that harmful bacteria and viruses cause the majority of foodborne illnesses, and some parasites and chemicals also cause foodborne illnesses: (i) Bacteria are tiny organisms that can cause infections of the gastroenteric tract. They may already be present in foods when they are purchased, such as meat, poultry, fish and shellfish, eggs, unpasteurized milk and dairy products, and fresh produce. Bacteria can contaminate food at any time during growth, harvesting or slaughter, processing, storage, and shipping; (ii) Viruses are tiny capsules that contain genetic material. Infections caused by viruses can lead to sickness. Viruses are present in the stool or vomit of people who are infected, and people can pass them to each other. The common sources of foodborne viruses include food prepared by a person infected with a virus, shellfish from contaminated water and produce irrigated with contaminated water; (iii) Parasites are micro organisms that live inside another

organism. They can spread through water contaminated with the stool of infected people or animals. Parasitic infections are relatively rare in developed countries, such as the United States; (iv) Chemicals, such as those found in certain types of wild mushrooms, pesticides on unwashed fruits and vegetables, and toxins in fish or shellfish that may feed on algae that produce toxins, can cause illness.

1.2. Foodborne and waterborne viruses++++

Any viruses that may be transmitted by contaminated food or water and is able to cause illness via the fecal-oral route are called foodborne and waterborne viruses. These food- and water-borne viruses usually shed and dispersed through stool and vomit after entering the human body, and human digestive system is their main target. Human norovirus (HuNoV), sapovirus, astrovirus, rotavirus, adenovirus, poliovirus, enterovirus 71, hepatitis A, and hepatitis E viruses are the most common food- and water-borne viruses. The properties and major symptoms of these viruses are presented in Table 1.

Table 1. The properties and major symptoms of the most common food- and water-borne viruses

Virus	Family	Genus	Genetic material	Envelope	Transmission mode	Signs and Symptoms
Hepatitis A virus	Picornaviridae	Hepatovirus	Single-strand Positive-sense-RNA	No	Food, water	Diarrhea, dark urine, jaundice and flu-like symptoms
Hepatitis E virus	Hepeviridae	Hepevirus	Single-strand Positive-sense-RNA	No	Water, food	Diarrhea, dark urine, jaundice and flu-like symptoms
Human norovirus	Caliciviridae	Norovirus	Single-strand Positive-sense-RNA	No	Food, water	Nausea, vomiting, diarrhea, abdominal cramping
Human sapovirus	Caliciviridae	Sapovirus	Single-strand Positive-sense-RNA	No	Food, water	Nausea, vomiting, diarrhea, abdominal cramping
Rotavirus	Reoviridae	Rotavirus	Double-strand RNA	No	Food, water	Vomiting, watery diarrhea, low-grade fever
Adenovirus	Adenoviridae	Mastadenovirus	Double –strand DNA	No	Water, food	Nausea, vomiting, diarrhea, fever, headache
Astrovirus	Astroviridae	Astrovirus	Single-strand Positive-sense-RNA	No	Food, water	Nausea, vomiting, diarrhea, fever, headache
Poliovirus	Picornaviridae	Enterovirus	Single-strand Positive-sense-RNA	No	Water	Flu-like symptoms, poliomyelitis
Enterovirus 71	Picornaviridae	Enterovirus	Single-strand Positive-sense-RNA	No	Water, food	Respiratory illnesses, fever and generalized neurological abnormalities

HuNoV has long been considered the most common prominent virus related to foodborne illness(26, 78). More than 95% of nonbacterial acute gastroenteritis caused by HuNoV (35, 78). According to the most recent statistical report, the CDC (2010) claims that approximately 23 million people suffer from norovirus-induced gastroenteritis each year in the United States. In addition, it should be emphasized that nearly 60% of estimated illnesses could be attributed to HuNoV alone (19).

The good news is that in contrast to bacteria, viruses are unable to amplify in food or water during transportation, processing, and storage because they are strict intracellular parasites and only replicate inside of living host cells (63). Unfortunately, all known food- and water-borne viruses can survive on human hands, dried human and animal feces, kitchen surfaces, floors, carpets, and even hospital lockers for a long time because they are non-enveloped viruses and extremely stable in food, water, and the environment(12). Their infection doses usually are small, only a few virus particles are possible to cause illness, and a large amount of virus particles can be shed through an infected person's stool (up to 1011). All of these reasons lead the disease to spread rapidly (62, 90). Compared to bacteria, most food-and water-borne viruses are much more resistant to heat, acidic pH, and disinfection. Therefore, current procedures to prevent bacteria infections in food processing, preservation, and storage may not be fully effective against viral pathogens (32, 62, 90). Importantly, because the products look, smell, and taste normal even after contaminated, it is impossible to notice the viral contamination in food or water (63). In addition, the most common method--molecular techniques--used to detect viruses is difficult to apply in foods because most foods are in a complicated matrix. And there is a lack of methodologies for detecting foodborne viruses in foods.

1.3. The virology of human norovirus

The Norwalk agent was the first virus that was identified as causing gastroenteritis in humans (40). The first documented outbreak of human norovirus (HuNoV) occurred in 1968 at an elementary school in Norwalk, Ohio(59). More than 50% of the students and teachers at Bronson Elementary School were reported to develop acute gastroenteritis. The same symptoms, including nausea and vomiting, were developed in 32% of home contacts of the students and staff. However, it was not until 1972 that Kapikian et al. first determined the etiology of this disease, and clearly linked this virus to an outbreak of diarrhea with the help of immune electron microscopy. This is where norovirus received its former name of Norwalk-like virus. Likewise, viruses discovered later were assigned names based on the locations of outbreaks (eg, Appalachicola Bay, Amesterdam, Desert Shield, Fort Lauderdale, Gwynedd, Hawaii, Idaho Falls, New Orleans, Southampton, Toronto) (101). The name of this virus was changed to norovirus after it was identified in numerous outbreaks with similar symptoms occurring on cruise ships and in many other settings. Some other names used to refer to norovirus are winter vomiting disease, viral gastroenteritis, food poisoning virus, small round-structured virus and the stomach flu(2, 59). However, in 2002, the International Committee on Taxonomy of Viruses approved the name norovirus (Norovirus for the genus). The illness caused by norovirus is normally called the winter vomiting disease, acute non-bacterial gastroenteritis, viral gastroenteritis, or stomach flu.

The human noroviruses belong to the Norovirus genus in the *Caliciviridae* family. They are non-enveloped, single-stranded positive-sense RNA viruses with a small round-structured viral particle ranging from 27-38 nm in diameter in human stool samples (74). As shown in Table 2, the *Caliciviridae* include six genera: *Norovirus* (e.g. Norwalk virus), *Vesivirus* (e.g. Vesicular exanthema of swine virus and Feline calicivirus), *Lagovirus* (e.g. Rabbit hemorrhagic disease virus and European brown hare syndrome virus), *Sapovirus* (e.g. Sapporo virus), and *Recovirus* (e.g. Tulane virus).

Table 2. Classification of family Caliciviridae

Genus	Species	Specific strains
Norovirus	Norwalk virus (NV)	Norwalk, Southampton, Desert shield, Chiba, BS5 kidneys, etc. (GI) Hawaii, Lordsdale, Camberwel, U201, Alphatron, etc. (GII) Bovine enteric calicivirus, Murine norovirus, Swine norovirus, etc.
Sapovirus	Sapporo virus (SV)	Sapporo, Manchester, Houston, Parkvlle, etc. Porcine enteric sapovirus
Vesivirus	Vesicular exanthema of swine virus (VESV) Feline calicivirus (FCV)	Feline calicivirus, Urbana, F9, Japanese F4, Vesicular exanthema of swine virus, etc. Bovine calicivirus, Primate calicivirus, San Miguel sea lion virus, etc.
Lagovirus	Rabbit hemorrhagic disease virus (RHDV) European brown hare syndrome virus (EBHSV)	Rabbit hemorrhagic disease virus
Recovirus	Tulane virus	Tulane virus

The outer shell of the caliciviruses particle composed by a highly stable protein capsid, which exhibits icosahedral symmetry, and it protects the genomic RNA. Unfortunately, many of caliciviruses are not cultivable or lack a suitable small animal model for study.

Based on the phylogenetic analysis, noroviruses are further classified into distinct genogroups. Currently, five genogroups of noroviruses (GI, GII, GIII, GIV, and GV) have been recognized. The majority of HuNoVs belong to genogroup II,

and genogroups IV and I also infect humans, while genogroups III and V contain bovine and murine strains, respectively (119). Within a genogroup, strains are further subdivided into different genotypes (Fig. 1). To data, there are at least 33 norovirus genotypes. In total, there are 19 genotypes assigned to GII noroviruses, which is the most prevalent genogroup of norovirus (42). More specifically, the most prevalent HuNoV strain belongs to genogroup II, gentotype 4 (GII.4) and is responsible for the majority of outbreaks of gastroenteritis worldwide (74). Fankhauser (1998) estimated that the predominant norovirus strains changes seasonally, and can differ significantly between outbreaks.

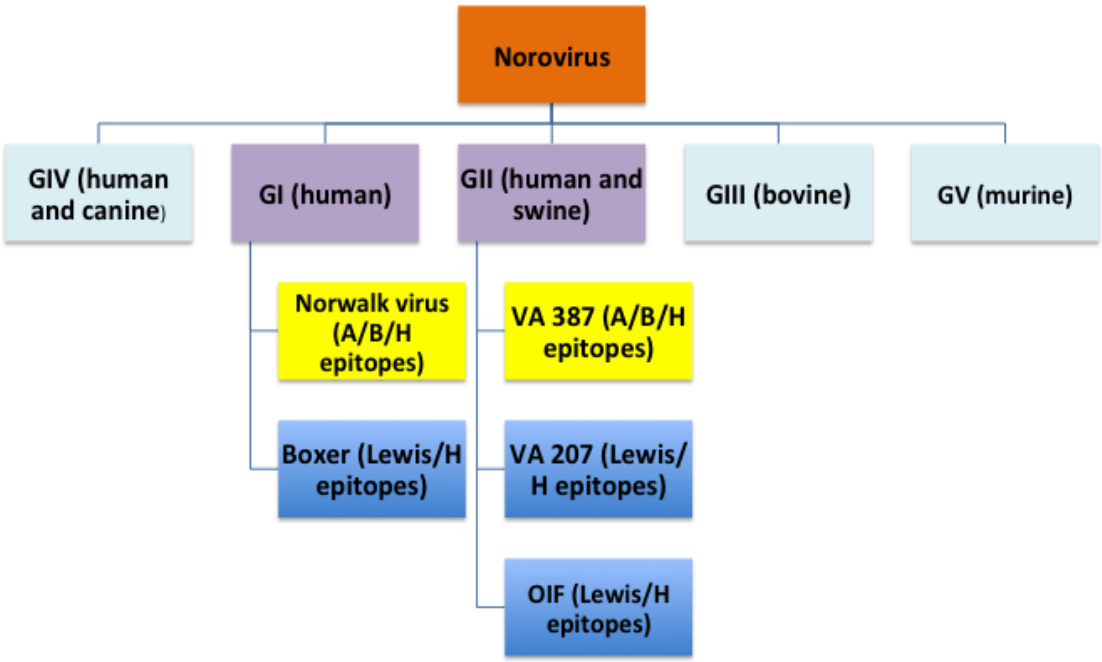


Figure 1. Major genogroups of norovirus

To date, there is no evidence to support that noroviruses are zoonotic. Although, Mattison et al. (2007) stated that they found HuNoV strains in cattle and swine by RT-PCR, this is may due to contaminators entering products through an external source during processing and the animals themselves were not infected with HuNoV. There is not any report released about animal strains causing illness in humans, suggesting that noroviruses are species specific.

1.4. Epidemiology and transmission of human norovirus

Noroviruses are considered the major viral etiologic agents responsible for the epidemic foodborne and waterborne viral gastroenteritis (13). The CDC claims that noroviruses were responsible for 96% of reported outbreaks of viral gastroenteritis from January 1996 to June 1997(24). Persons who infected by HuNoV will shed HuNoV through stool and vomit throughout the infection, starting from the incubation period (1-3 days) and lasting even after full clinical recovery. More importantly, in the immunocompromised, the elderly, and children, the duration of viral shedding is increased (45, 87). It has been estimated that the stool of an individual with an active norovirus infection may shed up to 100 billion virus particles per gram of feces (20). The disease caused by norovirus has a high rate of transmission because norovirus infections are highly contagious, with only a few particles (≤ 10) sufficient to cause the disease(32, 100). Outbreaks occur in a wide variety of closed or semi-closed communities (e.g., nursing homes, hospital wards, day-care centers, cruise ships, restaurants, schools, swimming pools, and even military installments) where large numbers of people are in close contact with each other (41, 87). These outbreaks

involve people of all ages, and target a number of high-risk groups, such as young children and elderly, travelers, soldiers and patients who are immunocompromised or have received organ transplants (40).

Noroviruses are important causative agents in community-based gastroenteritis (40). As shown in Figure 2, of the 660 norovirus-associated outbreaks laboratory confirmed by CDC from 1994 to 2006, 35.4% (234 cases) happened in long-term care facilities, 31.1% (205 cases) happened in restaurants, parties and events, 20.5% (135 cases) happened in cruise ships and vacations, and 13.0% (86 cases) happened in schools, child care centers and communities (CDC, 2011).

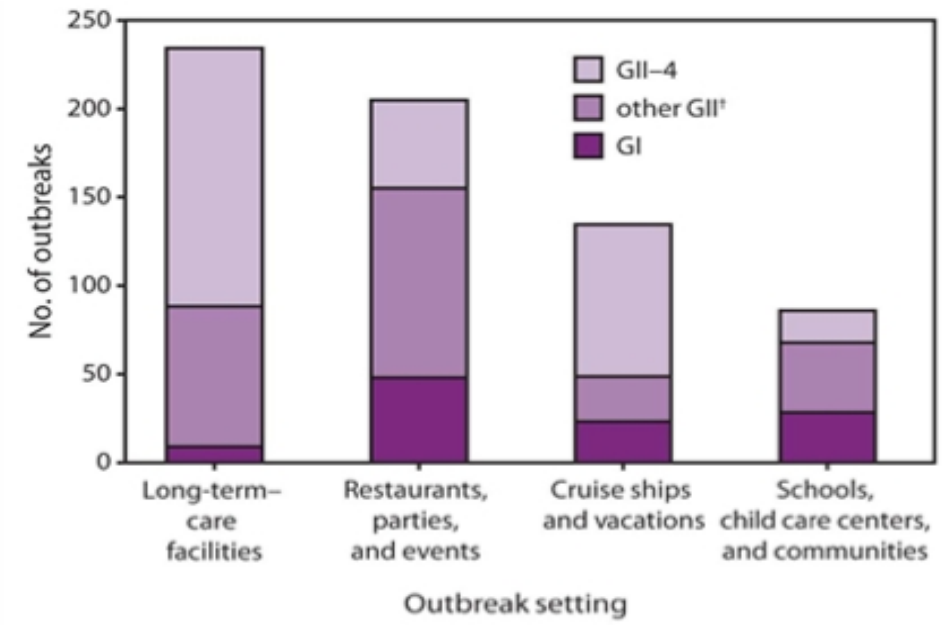


Figure 2. Number of norovirus outbreaks confirmed by CDC by setting and genotype in United States from 1994 to 2006

As shown in Fig. 3, HuNoV can transmit through contaminated food, water, and fomites, or directly transmitted from person to person. The primary route of viral transmissions for noroviruses is fecal-oral transmission (4, 62). Transmission through infectious vomit or feces either by direct contamination or by indirection contamination may also account for the rapid transmission of the virus in closed settings (111). Contamination of food or water accounts for the primary source of infection, and person-to-person spread further disseminates the norovirus outbreak (10). For instance, it was reported that out of eleven outbreaks in New York State, the suspected modes of transmission were person-to-person in seven outbreaks, foodborne in two, waterborne in one, and unknown in the last (49).

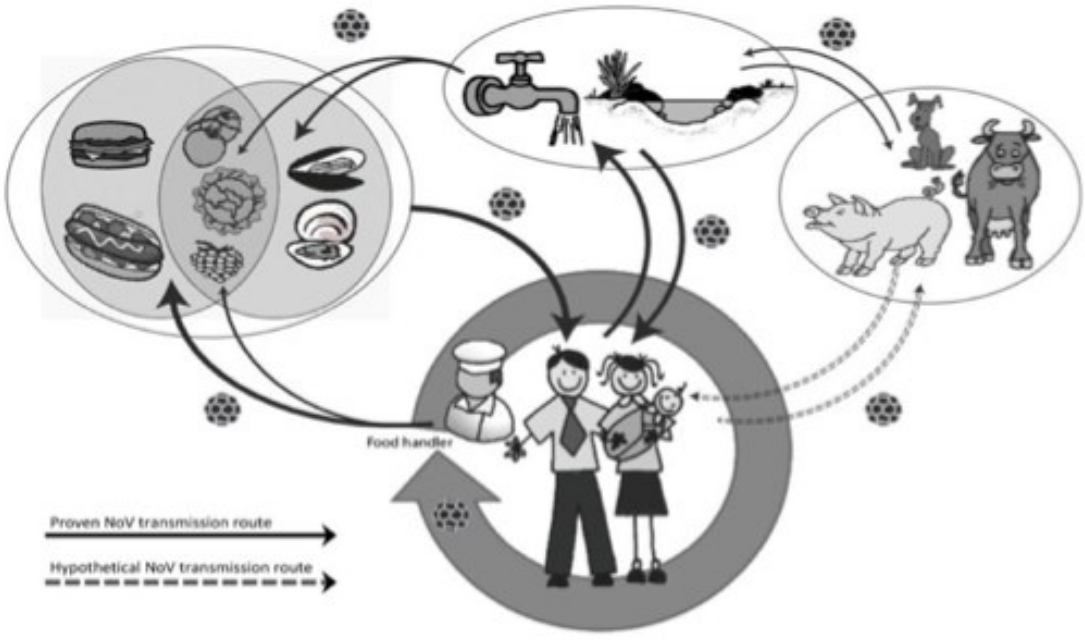


Figure 3. Transmission of food- and water-borne viruses

Several characteristics of noroviruses contribute to the high prevalence of norovirus and their persistence. First, HuNoV has a low infectious dose (approximately 18 to 1000 viral particles). And the virus is extremely stable in the environment. The commonly used disinfectants (e.g. alcohols and quaternary ammoniums) are unable to inactivate this virus (31, 32, 77). More importantly, the virus remains viable for several months after shedding (10, 62, 100). Secondly, up to 30% of exposed persons start viral shedding before the onset of illness (4), and the virus shedding will continue for prolonged periods even after illness stops, therefore many previous patients become asymptomatic individuals who still have the potential to spread disease (43, 87). All these factors increase the potential risk of secondary spread (4). Thirdly, because of the great diversity between different norovirus strains and the lack of complete cross-protection, repeated infections could occur throughout life. Fourthly, the norovirus genome easily undergoes mutation, which in turn causes antigenic shift and recombination. This may result in the evolution of new strains that are able to infect susceptible hosts (35). Finally, although short-term immunity to these agents has been supported by early volunteer studies, there is no long-term immunity to norovirus (35). Based on the characteristics above, National Institute of Allergy and Infectious Diseases (NIAID) classified HuNoV and other caliciviruses as category B priority biodefense agents.

1.5. Clinical features of human norovirus

In order to summarize a description of clinical symptoms, many volunteer studies proceeded as well as studies of outbreaks (4, 43, 87). The virus begins to replicate within the small intestine after a person becomes infected with norovirus. Symptom development can be either gradual or abrupt. The principal symptom that HuNoV infected adults develop is diarrhea, however nausea and vomiting are more often in children. Diarrheal stool is non-bloody, lacks mucus, and may be loose and watery (74). In 25-50% of infected persons, symptoms accompanying the infection most commonly are headache, fever, chills and myalgia. Moreover, a low-grade fever (101-102 F) was found in about half of infected individuals (103). The average incubation period is 24-48 hours, and symptoms typically resolve in 2-3 days(74). Kaplan et al. (1982) proposed criteria for the identification of norovirus-associated outbreaks of nonbacterial gastroenteritis based on these characteristics (Table 3). These criteria are still valid, having a sensitivity of 68% and specificity of 99%, when modern diagnostic methods are used(106).

Table 3. Kaplan criteria for identification of noroviruses as the cause of nonbacterial gastroenteritis outbreaks

Stool culture negative for bacterial pathogens
Vomiting in > 50% of cases
Mean/median incubation period of 24-48 h
Mean/median duration of illness of 12-60 h

Data from (60, 106)

Although for healthy individuals the illness associated with HuNoV is considered self-limiting, parenteral fluid therapy and even hospitalization are needed for some severe cases (60, 63). Currently, more fatal cases occur in children, the elderly, the immunocompromised, and even infants. For example, out of the 23 million annual cases of norovirus infection, there are about 300 cases that are fatal, most of which occur in infants, children, the elderly, the malnourished, or the immunocompromised (17, 41). In the United Kingdom, an estimated 80 deaths from norovirus infection occur each year among persons older than 64 years of age(45). Recent reports have suggested possible associations of norovirus infection with necrotizing enterocolitis in newborns, with benign seizures in infants, and with exacerbations of inflammatory bowel disease in pediatric patients; further study is needed to confirm these links (107). Importantly, the duration of virus shedding was found increased in the immunocompromised, the elderly, and children (45, 87).

1.6. Molecular biology of human norovirus

1.6.1. Human norovirus genome

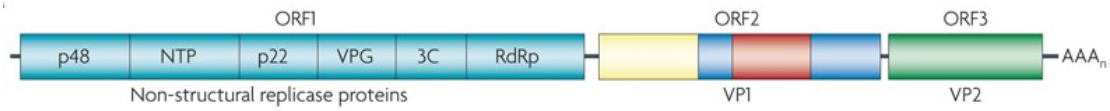


Figure 4. Genome of human norovirus (adapted from Donaldson 2010)

As shown in Fig. 4, the genome of HuNoV is positive-sense single-stranded RNA approximately 7.5-7.7 kb in length and encodes three open reading frames (ORFs) (56). The first ORF (ORF1) encodes a ~200kDa nonstructural polyprotein, which can be proteolytically cleaved into six non-structural proteins including N-terminal protein (designated p48 for Norwalk virus), NTPase, 3A-like protein (designated p22 for Norwalk virus), VPg, viral protease ($3CL^{pro}$), and RNA-dependent RNA polymerase (RdRp) (74). Although the absolute number of mature nonstructural proteins and functional precursors is not clear yet, the many essential roles of these six non- structural proteins in virus life cycle are shown in Table 4. The second ORF (ORF2) is approximately 1.8kb in length and encodes the 58-60 kDa major capsid protein VP1, and ORF3 (~0.6kb in length) encodes the 22-29 kDa minor structural capsid protein VP2 (44).

Table 4. Function of norovirus nonstructural proteins

Proteins name	Function(s)
P48	Functions as scaffolding protein during the formation of replication complex; Inhibits the expression of host protein to the cell surface
NTPase	Has NTPase activity
P22	Inhibits host cells' secretory pathways; Play an important role in the formation of replication complex
VPg	Primes viral RNA replication following its uridylylation; Recruits host translation initiation factors
3CL ^{pro}	Functions as protease
RdRp	Generates uridylylated VPg; Involved in viral genomic RNA replication
VP1	Major capsid protein
VP2	Minor capsid protein, involved in packaging the viral genome and stabilizes VP1

1.6.2. Major capsid protein VP1 of human norovirus

The ORF2 encodes a 58-60 kDa major capsid protein (VP1), which normally ranges from 530-555 amino acids in length. VP1 is the major component of the outer shell of the human NoV virion, protecting the genomic RNA from degradation. VP1 folds into two major domains—the shell domain S and the protruding domain P. During the virus life cycle, VP1 plays many essential roles. The histo-blood group antigen (HBGA) on the host cells can be bound by VP1 to mediate virus entry. Prasad

et al. (1999) demonstrated that antigenicity and strain specificity is determined by VP1 protein. In fact, the classification of norovirus genogroups and genotypes is based on the diversity of VP1 protein. VP1 may play many important roles in the virus life cycle such as uncoating, assembly, and release (44). In addition, VP1 protein is the host protective antigen that is responsible for eliciting neutralizing antibody, cellular, and mucosal immunities (11). In 1993, Jiang et al. first found VP1 assembles into HuNoV virus-like particles (VLPs) when expressed alone in E.coli. These VLPs can also be produced efficiently in other expression systems such as mammalian cells, insect cells, and yeast (76). Although these VLPs are structurally and antigenically similar to native virions, they do not contain the viral genetic material (RNA). In addition, VLPs can be expressed and purified in relatively high yield. Thus, researchers can use VLPs as important tools to study the structural, immunological, and biochemical properties of HuNoV.

(A) (B)

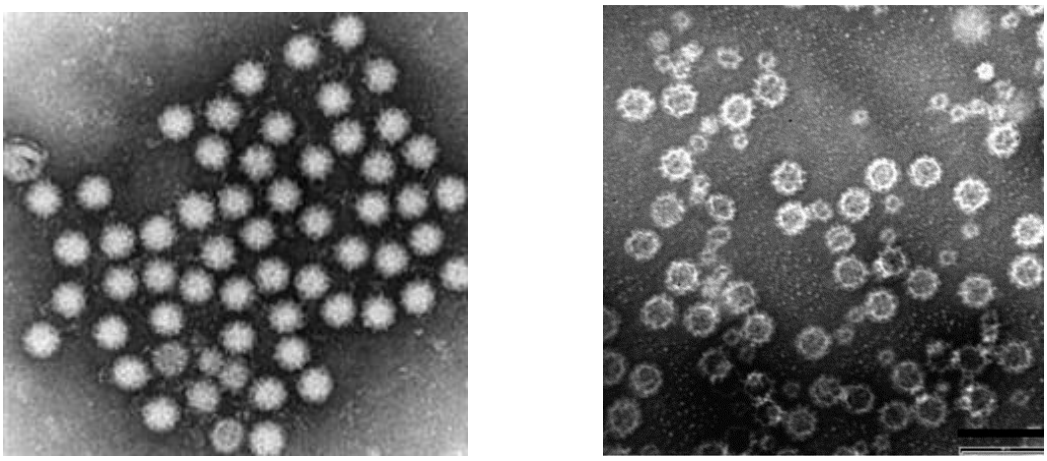


Figure 5. (A) The morphologies of human norovirus and (B) human norovirus virus-like particle under electron microscope

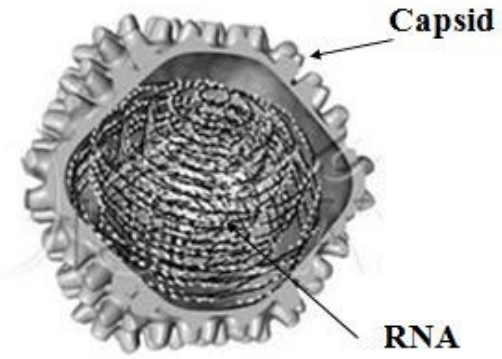


Figure 6. The structure of norovirus virion

1.6.3. Minor capsid protein of VP2 of human norovirus

VP2 is the minor capsid protein composed of 208-268 amino acids with a molecular weight of about 29kDa, and exhibits extensive sequence variability in different strains. VP2 is essential for the production of infectious virus although is not necessary for virus-like particle assembly (44). VP2 is a basic protein that has an isoelectric point is larger than 10, and this chemistry suggests that VP2 may be involved in RNA binding and viral genome packaging(44). VP2 also regulates the synthesis of VP1 protein because the presence of VP2 leads to an increased expression of VP1(11). Moreover, VP1 can be stabilized and protected from disassembly and protease degradation by the presence of VP2 (11). Fig. 7 shows the proposed model for the role of VP2 in stabilizing VP1.

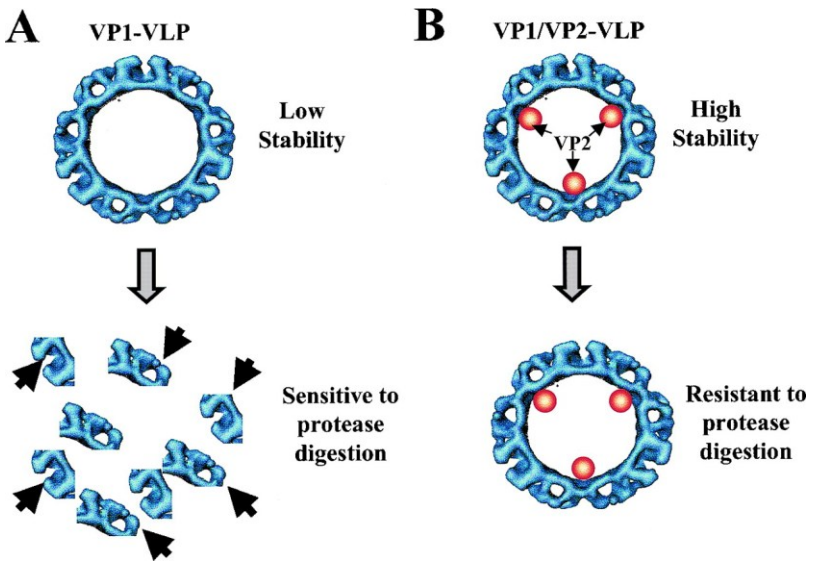


Figure 7. Proposed model for the mechanism of the VP2 stabilizing effect on VP1 protein (Adapted from Bertolotti-Ciarlet et al. 2003)

1.7. Proposed life cycle of human norovirus

Because HuNoV cannot be grown in cell culture, its life cycle is poorly understood. Most of our understanding of the life cycle of HuNoV is based on the studies of surrogates such as VLPs, murine norovirus, and feline calicivirus. The proposed HuNoV life cycle is summarized as following (Fig.8):

(1) Attachment: In most cases, specific attachment proteins on the surface of viruses bind to specific cellular receptors. HuNoV utilizes HBGAs as functional receptors in a strain-dependent manner. The P domain of the VP1 protein mediates this.

(2) Penetration: The virus or at least its nucleic acid must enter the cell once the virus bounds to the cell membrane. Animal viruses do this primarily through one of two mechanisms (Endocytosis or Direct Membrane Fusion).

(3) Uncoating: The viral capsid is released from the virion and the viral genomic RNA is delivered into the cytoplasm of the infected cell during or after penetration.

(4) Replication and gene expression: Like most RNA viruses, the replication of norovirus happens in the cytoplasm. The viral RNA dependent RNA polymerase (RdRp) is responsible for genome replication and uridylylation of VPg. First, the positive-sense RNA genome is recognized as a template for the RdRp to synthesize negative-sense RNA. Then the positive-sense RNA genome is synthesized using the negative-sense RNA as a template. Norovirus produces subgenomic RNA that only contains the VP1 and VP2 genes during replication. The VPg is covalently linked to the 5'-end of genome that recruits host translation initiation factors to synthesize viral proteins. The three ORFs are translated into a large polyprotein, VP1, and VP2. Subsequently, the polyprotein is cleaved into six non-structural proteins.

(5) Assembly: Once the newly synthesized viral genomes and capsid proteins have been produced, they are assembled into new virions.

(6) Release: The mature virions escape from the cell by lysing the cells.

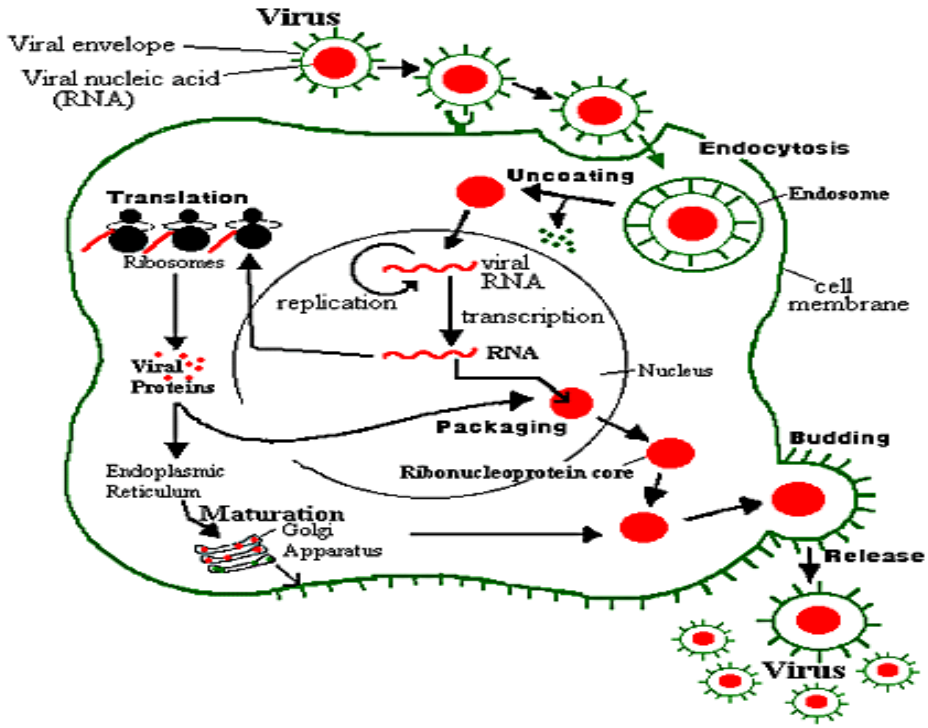


Figure 8. Proposed life-cycle of norovirus. The cycle is from infection of a cell by a single virus to release of thousands of offspring.

1.8. Human norovirus receptors and host susceptibility

Viruses must bind to cellular receptors to initiate an infection. Histo-blood group antigens (HBGAs) have been identified as functional receptors for HuNoV (53, 54, 98). In saliva, blood, milk, and contents of the intestine, HBGAs present as free oligosaccharides (98). There are three major families of HBGAs, namely Lewis, ABO, and secretor families, and all of them are involved in binding norovirus. The interaction between virus-like particles (VLPs) and HBGAs receptor has been

extensively studied. It was found that HBGAs receptors bind with the viral capsid protein VP1. Specifically, it has been demonstrated the amino acid residues in the primary site of receptor recognition--P domain of VP1 protein--are responsible for the specificity of receptor binding (98).

Many historical puzzles about HuNoV infection have been resolved due to the discovery of HBGAs as norovirus receptors. In a human volunteer study, it was found that individuals with an O blood type were easily infected by some norovirus strains, while those with a B blood type had the lowest risk of infection (52). Thus, we can assume that the type of human HBGA receptors decide the susceptibility of an individual to HuNoV. Further researches show the volunteers with a B blood type never became infected with certain norovirus strains following challenge simply because their intestinal epithelium lack the matched viral receptors(74). The observation that some individuals with a high level of antibodies against norovirus were even more susceptible to norovirus challenge than those with no or lower levels of antibodies may due to the specific virus-receptor interactions (82). Although acquired immunity is also involved in resistance to norovirus infection, the receptor specificity plays an important role in host susceptibility can be supported by these studies suggest.

1.9. Major challenges for human norovirus research

Noroviruses are highly contagious, extremely stable, resistant to common disinfectants, and have a low infectious does, thus are classified as category B biodefense agents by the National Institute of Allergy and Infectious Disease (NIAID). Unfortunately, the research on HuNoV has been severely hampered. Molecular biology, gene expression, replication, pathogenesis, and immunology of HuNoV are poorly understood currently. We also don't understand the stability and susceptibility of HuNoV to food processing technologies. This is due to two major challenges in HuHoV research. First, in fact HuNoV cannot be grown in cell culture. This cause researches relying on cell culture cannot be conducted. Second, the lack of small animal models for HuNoV infection. So, the development of proper surrogates is necessary for HuNoV studies.

Since the discovery of HuNoV, numerous efforts have been devoted to cultivate this virus, however an *in vitro* cultivation system for HuNoV has not been established so far. Duizer et al. (2004) created an *in vitro* cell culture system that mimics the intestinal epithelium using gastric cells, duodenal cells, and small intestinal enterocyte-like cells to systematically evaluated a variety of cell lines and laboratory methods to cultivate HuNoV. Unfortunately, they failed to cultivate HuNoV in all of the cell culture combinations. Using macrophages or dendritic cells that support murine norovirus to replicate also failed in HuNoV replication (71). Straub et al. (2007) first reported that HuNoV could infect and replicate in a physiologically relevant 3-dimensional (3-D) organoid model of human small intestinal epithelium. The norovirus RNA was detected at each of the five cell

passages for HuNoV genogroup I and II, and the cytopathic effect (CPE) also be found. These results are very encouraging; however, the amount of newly synthesized viruses and the level of virus replication have been questioned (22). In response to this question, Straub et al. (2007) claimed that norovirus titer in their culture system did increase. However, they also underlined that both virus strain and multiplicity of infection affect the magnitude and time course of these increases.

Recently, an *ex vivo* culture system was reported to cultivate HuNoV by using freshly collected human duodenal tissues that mimics the primary site of viral replication in vivo (73). The RNA levels in cell-free culture supernatants were measured by real time RT-PCR, and they found the viral genomic increased over time. Notably, HuNoV displays an obvious tropism for glandular epithelial cells revealed by *in situ* hybridization of viral RNA and immune-histochemical staining of VP1 as well as newly synthesized viral protease. It appears that *ex vivo* culture can support key stages ranging from virus adsorption and internalization to viral RNA replication and protein synthesis during complete HuNoV replication. However, the robustness of these cultivation systems is remains to be determined.

1.10. Human norovirus virus-like particles (VLPs)

HuNoV VLPs are not infectious due to the lack of viral genomic RNA. However, they are antigenically and morphologically similar to native virions. There are two major advantages for using VLPs to study human NoV. First, VLPs can be produced efficiently and easily by expression of VP1 in insect cells or mammalian

cells. Second, VLPs possess all the authentic information about HuNoV's structure, antigenic sites, and receptor binding activities. Therefore, in the researches to study the epidemiological, immunological, structural, and biochemical properties of HuNoV, the VLPs can be used as an important alternative tool. The damage of receptor binding activity would be lethal to this virus. Hence, the virus survival can be showed through VLPs' receptor binding. Alternatively, electron microscope and SDS-PAGE can be used to examine the damage of VLPs. The gamma irradiation can disrupt the structure of VLPs and degrade VP1 protein (36). In addition, the capsid of HuNoV shows a similar stability compared to MNV-1 after exposure to gamma irradiation (36). VLPs also can be used as a surrogate to study the interactions of norovirus with high-risk foods, such as fresh produce and seafood.

1.11. Vaccines against human norovirus

Vaccination is the most effective strategy to protect humans from infectious diseases. However there is no FDA approved vaccine is available for HuNoV currently. Because norovirus cause significant health, economic, and emotional burdens to humans, it is urgent to develop an effective vaccine against HuNoV. Since Jiang et al. (1992) first found self-assembled VLPs that yielded through the expression of VP1 alone in cell culture are structurally and antigenically similar to native virions, most HuNoV vaccine studies have focused on VLPs. To date, HuNoV VLPs have been obtained successfully through using E. coli, yeast, insect cells, mammalian cell lines, tobacco, and potatoes. Immunizing with VLPs orally or

intranasally successfully induce variable humoral, mucosal, and cellular immunities in mice (6). But, no protection data is available due to the insufficient to use mice as an animal model for challenge assay. Instead, Souza and Menira (2007) used gnotobiotic pig as an animal model. Their result showed that VLPs-based HuNoV vaccine provided protection to the challenge of homologous GII.4 HuNoV strain.

In 1999, Ball and colleagues performed the first clinical study to demonstrate that baculovirus-expressed HuNoV VLPs were safe and immunogenic in humans when administered orally (5). Tacket et al. (2000) performed a human volunteer study of transgenic potato-based VLP vaccine. Nineteen of 20 (95%) volunteers who received transgenic potato-based VLP vaccine developed significant increase in the number of specific IgA antibody-secreting cells. Four of 20 (20%) volunteers developed specific serum IgG and specific stool IgA was found in 6 of 20 (30%) volunteers. Overall, 19 of 20 human volunteers developed an immune response in this study. In the US, LigoCyte Pharmaceuticals Inc. licensed two VLP-based vaccine candidates, dry powder formulation for intranasal delivery and liquid formulation for intramuscular delivery. And they performed human clinical trials for these VLP-based vaccines. Compared to those who received the placebo, there were significant reductions in clinical norovirus illness, infection, and severity of illness in individuals who received vaccine (LigoCyte Pharmaceuticals Inc., 2010). Moreover, for volunteers who received the dry powder VLP vaccine, the risk of illness was reduced by 47% after exposure to HuNoV (LigoCyte Pharmaceuticals Inc., 2010). Although these data suggest that a VLP-based vaccine is a promising vaccine candidate against HuNoV, there are some limitations. It is time consuming and expensive for VLPs preparation, it usually requires a high dosage of VLPs and multiple boosters for

immunization. Also, because VLPs are actually proteins, nonreplicating immunogens, the duration of antigen stimulation may be limited (74).

1.12. Antiviral drugs against human norovirus

Normally, norovirus-associated illness is usually self-limited, and no treatment is needed. However, there are many lethal cases due to HuNoV infection were reported in infants and young children in some developing countries. So effective anti-viral drugs may be necessary and beneficial because it has a high mortality rate in some severe cases. Unfortunately, no antiviral drug is currently available for HuNoV. Approaches to screen potential antiviral drugs are very limited because this virus is uncultivable so far.

In 2007, a saliva-based receptor binding assay was been developed by Feng and Jiang to screen a compound library for inhibition of norovirus VLPs binding to HBGA receptors. Among the 5,000 compounds screened, the activity of VLPs binding the A antigen was been inhibited by 153 compounds. Importantly, 14 of the 153 compounds revealed strong inhibition, with a 50% effective concentration less than 15 μM (74). Zhang et al. (2011) screened 50 Chinese herbal medicines through a similar assay, and they found two medicines (Chinese Gall and Pomegranate) were highly effective in blocking norovirus-receptor binding. More interestingly, the authors identified Tannic acid in these medicines as a strong inhibitor in binding of norovirus P particles to both A and B saliva with a half maximal inhibitory concentration (IC50) of 0.1μM. Although the antiviral drugs that target the first step

of viral infection, the virus-receptor binding, is promise to be developed based on these studies, no clinical data is available to evaluate the effectiveness of these compounds against norovirus infection.

1.13. Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) belongs to the Rhabdoviridae family, and it is an enveloped virus. In the US, there are two serotypes of VSV, namely VSV-New Jersey (VSV-NJ) and VSV-Indiana (VSV-IN). Under electron microscope, the VSV virions show bullet-shaped (Fig. 9B). Cattle, horses, and pigs are the main infected host. And it commonly causes an acute disease, including symptoms such as vesicles and ulcers around the mouth, in the teats and coronary bands, as well as fever and excessive salivation(72). Although the disease is rarely fatal, it will result in huge loss of milk production and weight gain. Currently, sheep, goats, llamas and humans who exposed to the infected animals also show to be infected. VSV infections in humans usually induce flu-like symptom or even being asymptomatic.

As shown in Fig. 9A, the genome of VSV is a non-segmented negative-sense RNA that encodes five structural proteins: glycoprotein (G), large polymerase protein (L), phosphoprotein (P), matrix protein (M) and nucleocapsid (N) protein.

N protein encapsidate the viral genomic RNA completely to form an RNase-resistant N-RNA complex that acts as a template for the RNA dependent RNA polymerase (RdRp) during RNA synthesis (1, 7, 8). The G protein is anchored in the envelope and plays an essential role in viral entry. L and P proteins catalyze the

replication of viral genomic RNA and the transcription of viral mRNA, and they are the major components of RdRP (39). Furthermore, the viral RdRp is tightly bound to N-RNA that results in the formation of the ribonucleoprotein (RNP) complex. Moreover, the M protein and lipid bilayer envelope surrounds this RNP complex. The robust growth of VSV in cell culture systems makes it a good virus model for research purpose.

(A) (B)

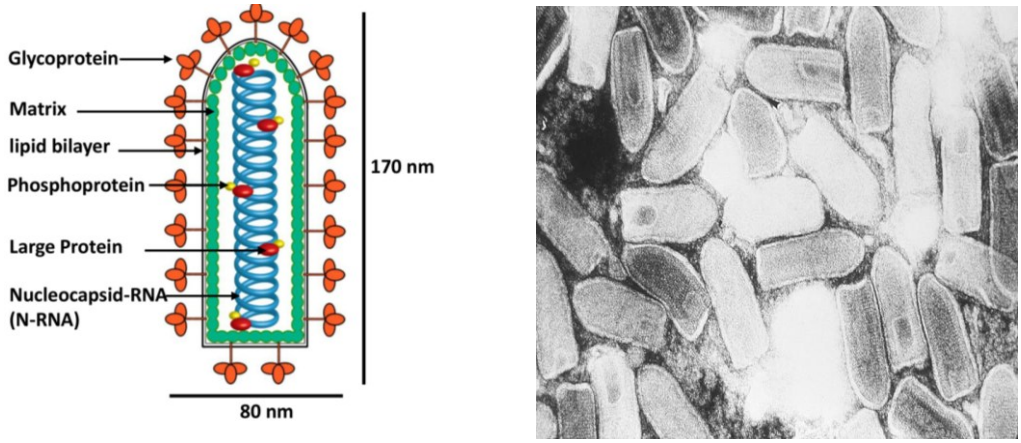


Figure 9. (A) The virion structure of vesicular stomatitis virus; (B) The morphologyof VSV under electron microscope

1.14. Passive immunization versus Active immunity

Active immunity refers to the process of exposing the individual to an antigen to generate an adaptive immune response. It may provide long lasting protection, but

it needs several days or even weeks to develop the response. In contrast, passive immunity, which need provide the ready-made antibodies from one individual to another (Figure 10), only lasts for a short period of time (several weeks to three or four months at most) after providing the active preformed antibodies, however it provides immediate protection (9).

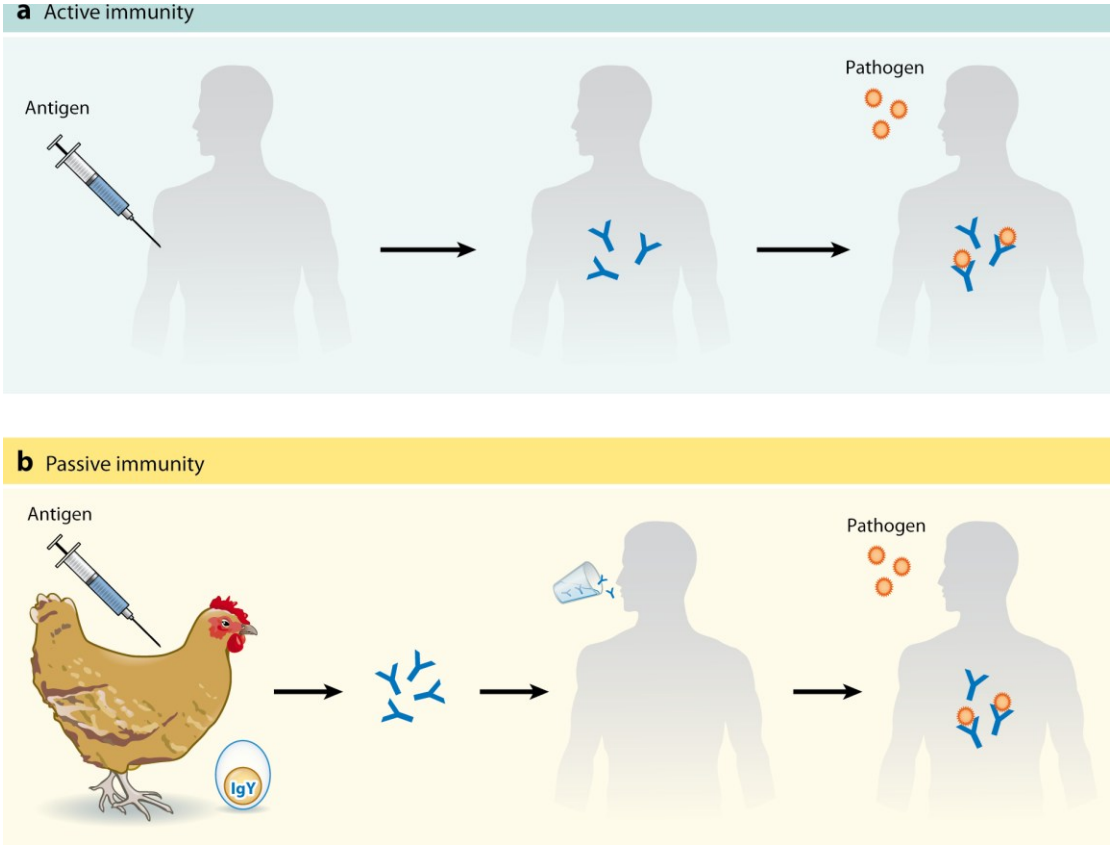


Figure 10. (A) Active immunity involves immunizing, or vaccinating, an individual with antigen to generate an adaptive immune response targeting the pathogen of interest. (B) In passive immunization, antibodies are isolated from another source (e.g., the yolks of immunized hens) and administered to susceptible individuals to provide pathogen-specific immunity. Adapted from Jennifer and Yoshinori et al. (2012).

Passive immunity can occur naturally. Neonates need get maternal antibodies from their mothers before the development of their own immune response due to the relatively immature immune system. In humans, the maternal antibodies--immunoglobin G (IgG)-- can be transferred from the mother to fetuses during pregnancy and the neonates obtain immunoglobin A (IgA) through the breast milk. Other mammals, such as cows, horses, pigs, sheep, and goats, obtain maternal

antibodies via colostrum, which are then transported across the intestinal epithelium of the neonates into circulation (25, 65, 75).

Passive immunity can also be acquired artificially. Unlike the natural passive immunity, the antigen-specific antibodies for acquired passive immunity need obtain from another source, such as an immune individual or animal (9). Due to the short period of protein provided by acquired passive immunity, it is necessary to repeat or continuous antibody administration. Therefore, large amounts of ready-made antigen-specific antibody are required, especially for orally administration.

High levels of antibodies specific for viral and bacterial pathogens can be recovered from immunized individual or from patient recovering from the infection and administered to non-immune individual, and the protective immunity has already been established through the administration of preformed specific antibodies in both humans and animals. Therefore, passive immunization is becoming a more and more interesting and attractive approach recently. In addition, the increasing number of new and antibiotic-resistant microorganisms, individuals with impaired immune systems who are unable to respond to conventional vaccines, and even diseases that are unresponsive to drug therapy leads more attention to passive immunization.

1.15. Hen egg yolk antibody--immunoglobulin Y (IgY)

Antibodies used as an indispensable tool in laboratory in various applications such as research, diagnostic and therapy because of their extreme properties to recognize small specific structures on other molecules. Antibodies presently available for these purposes are mostly mammalian monoclonal or polyclonal antibodies. Nowadays, rabbits and mice are the most classical chosen laboratory animals for the production of polyclonal and monoclonal antibodies, respectively. Two prerequisite steps are needed for antibodies preparation: immunize animals first, and then repeated bleeding or scarifying for spleen removal. Both of these steps will causes distress to the animals.

Klemperer (1893) first demonstrated that the immunization of a hen resulted in the transfer of specific antibodies from the serum to the egg yolk. Although hens' eggs have long been recognized sources of nutrients, there was no scientific application for egg yolk antibodies over a hundred years. However, since the 1980s Klemperer's results attracted a great attention when the scientific community set a matter of serious ethical concern for animal welfare. Using laying hens for antibody production only need egg collecting which replace the painful and invasive blood sampling or scarifying. Moreover, based on the weight of antibody produced per animal, antibodies productivity in laying hens is nearly 18 times greater than that in rabbits (92). Therefore, a refinement and a reduction in animal use.

In 1973, Leslie and Martin demonstrate that three classes of antibody can be found in the chicken: Immunoglobulin Y (IgY), Immunoglobulin A (IgA), and Immunoglobulin M (IgM). IgY is functional equivalent to immunoglobulin G (IgG) in mammals During egg formation, IgY in the serum is selectively transferred to the yolk to passively protect the developing chick via a receptor specific for IgY translocation on the surface of the yolk membrane (80, 99), whereas IgA and IgM are deposited into the egg white (89)(Figure 11).

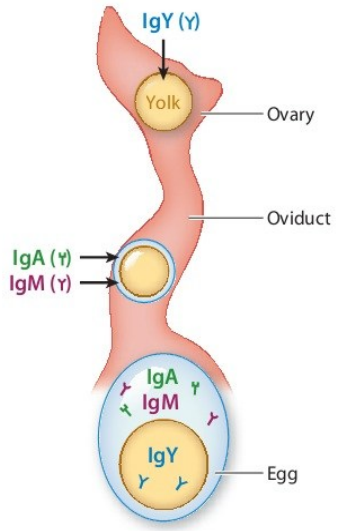


Figure 11. During egg formation, IgY (blue) is transferred from the blood to the egg yolk through receptors specific for IgY translocation. IgA (green) and IgM (purple) are later deposited into the egg white in the oviduct. Adapted from Hatta et al. (2008).

IgYs have been used in many diagnostic and biomarker discovery applications because the differences of immunoreactivities between IgY and IgG. In addition, the use of IgY for passive immunization applications has attracted many researchers. Antigen-specific IgY can be produced on a large scale from eggs laid by chickens immunized with selected antigens (48). Its effectiveness in preventing or treating infectious diseases caused by various pathogens has been demonstrated by many researches that focus on its use for passive immunization, especially for enteric pathogens (Table 5).

Table 5. Effect of passive immunization by enteric pathogen-specific IgY in humans and animals

Pathogen	Target species	Effects	**Reference**
Escherichia coli	Pigs	Curing diarrhoea affected piglets in a field study	(112)Wiedemann et al., 1991
		Preventing K88+, K99+, 987P+ ETEC infection in neonatal piglets	(114)Yokoyama et al., 1992
		Protecting pigs challenged with K88+ ETEC from E. coli-induced enterotoxernia	(115)Yokoyama et al., 1993
	Cattle	Protected against K99+ E. coli infection in calves	(55)Ikemori et al. 1992
		Reduced O157:H7 fecal shedding in feedlot steer	(30)DiLorenzo et al. 2008a
		Inhibited growth and internalization of O111 and enhanced uptake by macrophages	(118)Zhen et al. 2008
	Humans	Reduced binding of O157:H7 in vitro and protected mice from toxin challenge	(109)Wang et al. 2010
Campylobacter jejuni	Broiler chickens	Inhibiting the faecal shedding of C. jejuni	(105)Tsubokura et al., 1997
		Reducing the faecal shedding of C. jejuni	Tsubokura et al., 1997
Salmonella	Calves	Preventing fatal salmonellosis in neonatal calves exposed with S. Typhimurium or S. Dublin	(116)Yokoyama et al., 1998b
	Mice	Preventing mice challenged with S. Enteriditis from experimental salmonellosis	(84)Peralta et al., 1994
Rotavirus	Cattle	Protected neonatal calves from BRV-induced diarrhea	(67)Kuroki et al. 1994, (108)Vega et al. 2011
		Preventing BRV-induced diarrhoea in neonatal calves	(34)Özpinar et al., 1996
	Mice	Preventing murine rotavirus (MRV)	(117)Yolken et al., 1988
		Preventing mice from HRV-induced diarrhoea	(47)Hatta et al., 1993
		Protecting mice from bovine rotavirus (BRV)-induced diarrhoea	(66)Kuroki et al., 1993
		Preventing HRV-induced gastroenteritis in mice	(33)Ebina et al., 1996
Porcine epidemic diarrhea virus (PEDV)	Pigs	Protected piglets against PEDV infection	(68)Kweon et al. 2000

Antibodies are usually administered in the feed via several forms: whole eggs powder, whole yolks powder, water- soluble fraction powder or purified IgY material (Raja et al. 2008). Figure 12 shows the schematization of the utilization of preformed antigen-specific IgY for passive immunization in poultry.

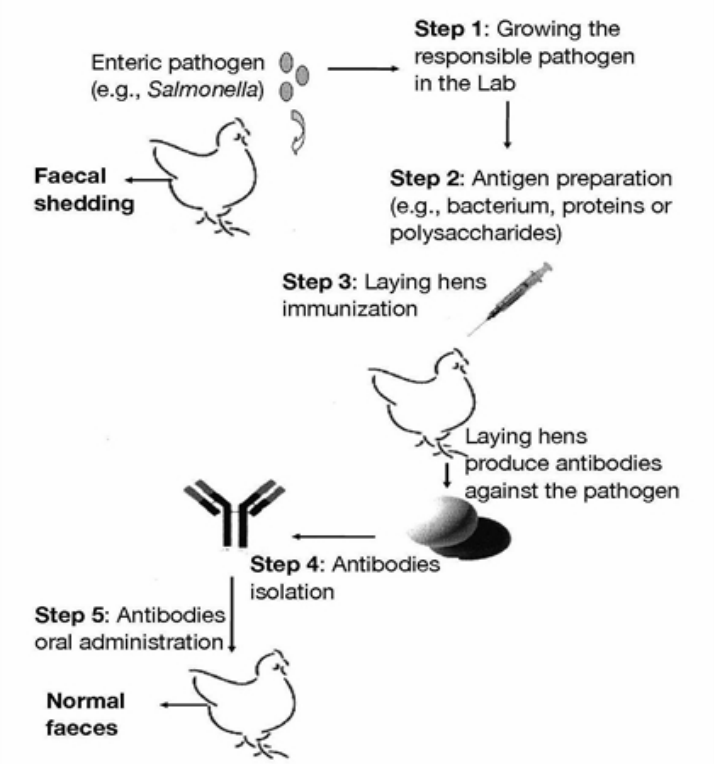


Figure 12. Summary of the egg yolk antibodies utilization for passive immunzation in poultry

1.16. Advantages of IgY

There are many advantages to use chickens for the production of polyclonal over production methods using mammals (Table 6). The most significant advantage is that the collection of eggs that contain antibodies is non-invasive. In the conventional

methods, the main source of antibodies is blood serum, thus animals are often sacrificed in order to collect a sufficient amount of blood to obtain antibodies. In contrast, production of antibodies through laying hens only requires the collection of eggs (70). In addition, the titer produced in chickens is high and long-lasting. A hen can be considered as a small "factory" for antibody production. From the time a hen starts to lay eggs, approximately 20 eggs can be produced per month, thus 300 eggs can be collected from a hen per year. The egg yolk in each egg is around 15 ml, and it contains 50–100 mg of IgY of which 2 to 10% are specific because of the high yolk antibody concentration (89). Therefore, one immunized hen produces more than 22,500 mg of IgY per year. That is equivalent to the production of 4.3 rabbits over the course of a year (91). The production of antibodies from yolk was nearly 18 times greater than that from rabbits. This reduces the need for frequent booster injections. Moreover, the maintenance costs for keeping hens are also lower than those for mammals such as rabbits (91). So, chickens present a much more economical source of large quantities of specific antibodies that can be used for passive immunization applications (93).

Table 6. Comparison of the characteristics of mammalian IgG and chicken IgY (Adapted from Schade et al., 2005)

Comparison items	Mammalian IgG	Chicken IgY
Main sources	Blood serum	Egg yolk
Antibody sampling	Invasive	Non-invasive
Antibody amount	200 mg IgG/bleed	50-100 mg IgY/egg
Sustained antibody production	From 60th day in rabbits	From 30th day in chicken
Frequency of collection	Every two weeks	Every day
Binding to Protein A/G	Yes	No
Activation of mammalian complement	Yes	No
Interference with mammalian IgG	Yes	No
Interference with rheumatoid factor	Yes	No

Another advantage is that due to the phylogenetic distance between chickens and mammals, fewer antigens are needed in order to induce an efficient immune response (70). And it is more successfully to produce antibodies against highly conserved mammalian proteins, which otherwise would not be possible in mammals (69). Carlander et al. (1999) demonstrated that chicken antibodies recognize different epitopes than mammalian antibodies, resulting in a different antibody repertoire.

IgY can easily be isolated from the yolk by precipitation techniques because it is the only a single class of antibody in egg yolk, which is different from mammalian serum (38). And IgY does not bind to protein A/G or interfere with mammalian IgG. It also does not activate mammalian complement or interact with mammalian Fc receptors that could mediate an inflammatory response (16). Collectively, IgY is

expected to become an effective natural food antimicrobial system and immunotherapeutic agent due to these unique biological attributes (113).

Lastly, the large-scale production of IgY technically is feasible due to the vaccination of hens and automated collection and processing of eggs is already carried out on an industrial scale (94). Therefore, compared with the traditional method of obtaining antibodies from mammalian serum, egg yolk provides a more hygienic, cost-efficient, convenient and rich source of antibodies.

When compared with antibiotics, using IgY can elicits no undesirable side effects, disease resistance or toxic residues and is much more environmentally friendly. Importantly, increasing evidences demonstrate the resistant organisms that caused by the reduction of antibiotic use may pass from animals to humans (Yegani & Korver 2010). It makes harder to treat infections, leading numerous studies to examine the use of IgY in both human and veterinary medicine.

1.17. Transfer of IgY into egg yolk

Antibodies (IgY, IgA and IgM) are transferred from hen to chick via the latent stage of the egg, and play an important role in immunological function for the relatively immuno- incompetent chick to resist various infectious diseases (Raja et al. 2008).

During egg formation, only IgY in serum is selectively transferred from the hen's circulatory system across the oolemma into the maturing oocyte in the ovarian follicle (88). On the surface of the yolk sac membrane, receptors specific for IgY

translocation promote the selective transportation of all IgY subpopulations presented by the maternal blood (79, 80, 104). In 2001, Morrison et al. had identified several regions within the antibody molecule important for its uptake into the egg yolk. Depend on their data, an intact Fc (Crystalizable (constant) region of antibody molecule) and hinge region were identified as necessary parts for this transport, but not the Fc-associated carbohydrate. In addition, Cγ2- Cγ3 domain (Figure 13) is responsible for IgY transport due to its interaction with the receptor. The IgY transovarial passage takes approximately 3-6 days (83).

Maternal IgA and IgM, in contrast, are deposited into the egg white in the oviduct along with the egg albumen secretion. IgY in egg yolk circulates in the blood of the chick through the endoderm of the yolk sac (83), and IgA and IgM in egg white are subsequently transferred to the embryonic gut through swallowed amniotic fluid (89). The concentrations of IgA and IgM in egg white are around 0.7 $mg.ml^{-1}$and 0.15 $mg.ml^{-1}$, which are relatively low. While IgY's concentration (8-25 $mg.ml^{-1}$) in egg yolk is considerably high (89).

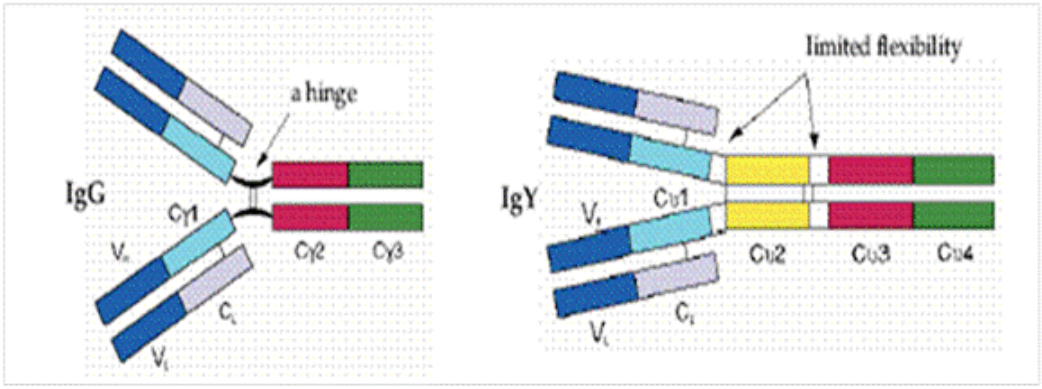


Figure 13. Structure of IgG and IgY (From Narat, 2003)

1.18. Characteristics of chicken immunoglobulin Y (IgY)

1.18.1. Structure and function of chicken IgY

IgY antibodies were thought to be similar to IgG immunoglobulins at first, while they are considered to be an evolutionary ancestor to mammalian IgG and IgE and also to IgA currently (110). Besides chicken IgY is functional equivalent to mammalian IgG, there are some profound differences between their structures. IgY has a molecular mass of 180 kDa that is larger than that of mammalian IgG (150 kDa) although both IgY and IgG molecule have two heavy (H) chains and two light (L) chains (Fig. 13).

There are 3 C refions (Cγ1, Cγ2, Cγ3) in IgG, while IgY has 4 C regions (Cγ1, Cγ2, Cγ3, Cγ4). The presence of one additional C region with its two corresponding carbohydrate chains logically results in a greater molecular mass of IgY compared with IgG. Thus, the molecular mass (50 kDa) of the H chain in mammalian antibodies is smaller than the H chain of IgY (67–70 kDa) (110). In fact there are other differences in their structures, include the hinged region of IgY is much less flexible than that in mammalian IgG. Davalos-Pantoja et al. (2000) also suggested that IgY is more hydrophobic compared with IgG. Finally, the isoelectric point of IgY is pH 5.7–7.6, whereas that of IgG lies between 6.1 and 8.5 (29, 97).

Different from mammalian IgG, IgY does not fix mammalian complement and does not interact with mammalian Fc and complement receptors (16). As well, IgY does not bind to protein A and protein G, or interfere with rheumatoid factor, so having false positives which is a problem with IgG-based mammalian assays can be

avoid on IgY-based immunoassay (29). These differences provide significant advantages to the application of IgY technology in many areas of research such as antibiotic- alternative therapy (16), diagnostics (34), and xenotransplantation (37).

1.18.2. Storage stability of IgY

Dried IgY preparations can be stored for five to ten years at 4 °C without significant loss of antibody activity, and the activity of IgY kept stable for six months at room temperature or one month at 37 °C (69). More importantly, the storage time for IgY solution at 4 °C can be extended from months to a few years under specified conditions, such as providing 0.02% NaN_3, 0.03%w/v thimerosal or 50 μg/ml gentamicin(91).

1.18.3. pH stability of IgY

The stability of IgY to acid and alkali has been studied under various conditions. It was found that IgY is relatively stable between pH 4 and pH 11, but displays a rapid reduction at pH 3.5 or lower and has obvious decrease at pH 12 or higher (95). At pH 3 IgY almost completely lost with irreversible change due to conformational changes and damage in the Fab portion including the antigen-binding site higher (96). However, adding addition stabilizers, such as sugars, complex carbohydrates and polyols can improve the stability of IgY (23).

1.18.4. Proteolysis stability of IgY

Hatta et al. (1993) demonstrated that almost all of the IgY activity was lost following digestion with pepsin, but after incubating with trypsin or chymotrypsin for 8 h, IgY remained 39% and 41% of activity, respectively. In spite of a definite breakdown of the polypeptides, the antigen- binding and cell-agglutinating activities of IgY retained after tryptic digestion. Unlike the trypsin digestion, the activities of IgY remained high with no definite cleavage of the IgY chains were observed after chymotryptic digestion (95). All these results suggest that IgY is relatively resistant to trypsin or chymotrypsin digestion even is fairly sensitive to pepsin digestion.

Moreover, pH and the enzyme/substrate ratio have a major impact on the stability of IgY against pepsin. The results of Shimizu et al. (1988) showed IgY had fairly resistance to pepsin and retained its antigen-binding and cell-agglutinating activities at pH 5 or higher. In contrast, both activities were lost at pH 4.5 or below. In 1993, Hatta et al. also observed the IgY behavior with pepsin under different incubation times and pH confirmed the susceptibility of IgY to pepsin at low pH.

The IgY molecule was completely hydrolyzed with pepsin at pH 2, leaving only small peptides. However, 91% and 63% of its activity retained after 1 h and 4 h incubation time with pepsin at pH 4, respectively.

1.18.5. Temperature and pressure stability of IgY

Shimizu et al. (1992) and Hatta et al. (1993) treated IgY thermally at various temperatures for different periods of time. They found IgY kept stable at temperature

60 to 70°C, however the binding activity between IgY and antigen decreased after increasing temperature and heating time. In addition, the IgY denatured seriously and decreased its activity when treated higher than 75°C(23, 95). There is no detectable inactivation of IgY by pressure up to 4,000 kg per cm^2, thus IgY is relatively stable to pressure(96).

1. 19. Production of IgY

1.19.1. Immunization of hens

Specific IgY development and production of large amounts of IgY in a cost-effective manner is key to its successful use for passive immunization. Several aspects of hen immunization will affect the immune response. Schade et al. (1996) demonstrated that the five mainly factors include the antigen (dose and molecular weight), the type of vaccine adjuvant, the route of application, the immunization frequency, and the interval between immunizations.

Antigen. Antigen will trigger the immune response when they contact with the organisms because the immune system recognize it as foreign object. Different results on the immune response and the antibody titer evoked based on the dose of antigen. In 1995, Hanly et al. demonstrated that too much or too little antigen might induce suppression, sensitization, tolerance or other unwanted immunomodulation. Schwarzkopf et al. (2000) found that good antibodies responses been elicited by the injection of antigen concentrations ranging between 10 μg and 1 mg, which in agreement with other research.

The type of antigens should also be considered. Antigen can be provided to the immune system as complex multi-antigens (e.g., bacteria, viruses and parasites) or as single antigens (e.g., proteins or polysaccharides). The most efficient immunogen is proteins, because the polymorphism of their structure and the differences existing between species and individuals (50). As a rule of thumb, the amount of protein antigen can be used ranges from 10 to 100μg(15, 102).

Peptides and polysaccharides antigens can also be used as antigen, but peptides need be coupled to carriers (e.g., bovine serum albumin or keyhole limpet haemocyanin). In addition, Goldsby et al. (2003) demonstrated that lipid and nucleic acids are not potent immunogens unless they are coupled to proteins or polysaccharides.

Adjuvant. The use of vaccine adjuvant influences the induction of high and sustainable egg yolk antibody titer. According to their chemical characteristics, their efficacy in stimulating the immune system, and their secondary side-effects, there are more than 100 known adjuvants can be chosen. The most effective adjuvant for antibodies production in laboratory animals is Freund's complete adjuvant (FCA). However, it may cause an eventual local tissue reaction in birds. Therefore, to avoid an eventual local tissue reaction, the Freund's incomplete adjuvant (FIA) becomes now the most commonly used adjuvant to produce egg yolk antibody. Because FIA is less efficient than FCA, Kapoor et al. (2000), Li et al. (2006) and Chalghoumi et al. (2008) preferred the use of a combination of the two adjuvants: FCA for the first immunization and FIA for the booster immunizations. Good results were achieved in these studies, and there is no adverse side effects were found.

Immunization routes. The intramuscular route is the most common route for antigen injection in hens for IgY production. Injection is usually performed in the breast muscle. Chicken can also be injected subcutaneously in the neck. Subcutaneous injection is more difficult to perform and can therefore cause more distress, especially for very young animals, thus, it may be preferable to inject intramuscularly into the breast muscle (92).

Immunization frequency and interval between immunizations. The type and dose of antigens decided the total number of immunizations, as well as the vaccine adjuvant employed. Yolk antibody titers should be checked 14 days after the last immunization, and at least two immunizations should be given. In 1996, Schade et al. demonstrated that the production of high levels of specific antibodies could be maintained up to year if booster immunizations were given during the laying period.

The success of immunization also depends on the interval between immunizations. The recommended interval is two to four weeks (102).

1.19.2. IgY extraction

Lipids and proteins are the major elements of egg yolk. Approximately one third of the yolk is lipid fraction, including triglycerides, phospholipids, and cholesterol. Stadeklman et al. (1977) demonstrated that proteins consist 15 to 17% of the yolk, and they can be separated into two main fractions by centrifugation, the granule (precipitate on centrifugation) and the plasma (clear fluid supernatant on centrifugation).

22% of the total yolk proteins are granules. And they composed of 70% high-density lipoproteins (HDL: α- and β-lipovitellins), 16% phosvitin (glycophospoprotein), and 12% low-density lipoproteins (LDL) (14). Plasma is about 78% of the total yolk proteins and composed of 86% of LDL and 14% of livetins. Livetins are divided into three classes: α-, β-, and γ-livetin, and they are water soluble, lipid-free globular glycoproteins. Bernardi et al. (1960) demonstrated the relative proportion of the three livetins in the yolk is 2:5:3, respectively.

IgY is the predominant fraction of γ-livetin (64). In order to get IgY, the lipoprotein is required to be removed first, and then recover the water-soluble fraction (WSF) followed by purification of the IgY from other livetins.

Several methods can be used for the removal of lipoproteins and the recovery of the WSF. In 1993, Akita et al. compared polyethylene glycol, dextran sulphate, and aliginates methods with water dilution methods in terms of yield, purity and activity of IgY. They found the water dilution method yielded the highest level (91%) and purity (31%) of IgY when compared with other methods. Followed by Polson method, both dextran sulphate method and phosphotungstic acid method were better methods with regard to IgY recovery (85).

The scale of extraction, quality of extraction, technology, and even cost effectiveness are the main factors during choosing a suitable IgY extraction method. For example, a simple and economical separation process is required if we want a large-scale production of IgY with high recovery and purity. In addition, in order to use IgY widely in food application, this process should require few chemicals.

After the recovery of the WSF, precipitation, chromatography or filtration can be followed to separate IgY (γ-livetins) from other water-soluble proteins (α- and β-livetins), and the remaining LDL(86).

CHAPTER 2

Efficient production of human norovirus-specific IgY in egg yolks by vaccination of hens with a recombinant vesicular stomatitis virus expressing VP1 protein

2.1. Abstract

Human norovirus (NoV) is the leading causative agent of acute nonbacterial gastroenteritis worldwide(74). Despite major efforts, there are no vaccines or effective therapeutic interventions against this virus due to the lack of a cell culture system and a small animal model. In this study, we developed a novel approach to produce human NoV-specific immunoglobulins (IgYs) from chicken yolks. White Leghorn chickens were immunized with a recombinant vesicular stomatitis virus (rVSV-VP1) expressing human NoV capsid protein (VP1) by intramuscular (im) route or combination of intramuscular and nasal drop route. After immunization, eggs were collected daily and IgYs were purified from each egg. It was found that human NoV-specific IgY produced by intramuscular vaccination was significantly higher than by the combined vaccination ($P<0.05$). At week 4 post-vaccination, human NoV-specific IgY reached 4.8 mg per yolk, which is approximately 3 times more than that in the combined vaccination group. ELISA and Western blot showed

that the egg yolk antibodies were specifically recognized by human NoV virus-like particles (VLPs), which are antigenically and structurally similar to native virions. Importantly, egg yolk antibodies specifically blocked the binding of human NoV VLPs to the histo-blood group antigens (HBGAs) (types A, B, and O), the functional receptors for human NoV. The purified IgYs were stable at temperature below 70 ^{o}C and at pH from 4 to 9. Taken together, these data demonstrated that intramuscular vaccination of rVSV-VP1 triggered a high level of human NoV-specific IgYs that were capable of blocking the interaction between human NoV VLPs and HBGA receptors. These results suggest that egg yolk may be a practical strategy for large-scale production of anti-human NoV antibodies for potential use in passive immunization against human NoV infection, as well as for therapeutic and diagnostic purposes.

2.2. Introduction

The virus family *Caliciviridae* contains five viral genera (*Norovirus, Sapovirus, Lagovirus, Vesivirus, and Recovirus)* that infect many different animal species including humans. Most of these agents are enteric pathogens whose replication and chief clinical manifestations are gastroenteritis and potentially life-threatening diarrhea. Examples of these viruses include human norovirus (NoV), porcine NoV, bovine NoV, human sapovirus, porcine sapovirus, and recently discovered Tulane virus. Human NoV is the major food- and water-borne virus that accounts for more than 95% of nonbacterial acute gastroenteritis worldwide, but this

percentage may be underestimated due to the large number of asymptomatic NoV infections and lack of proper detection methods (35, 62, 78). In addition, human is responsible for over 50% of the outbreaks of foodborne illnesses in the USA. The symptoms often involve projectile vomiting, diarrhea, nausea, and low-grade fever (2, 35, 62). Human NoV is transmitted primarily through the fecal-oral route, either by direct person-to-person contact or by fecally contaminated food or water. Although human NoV infection is usually self-limited disease, it is highly contagious, and only a few particles (usually less than 10) are thought to be sufficient to cause an infection (32, 35, 62). Currently, the National Institute of Allergy and Infectious Diseases (NIAID) classify human NoV and other caliciviruses as category B priority biodefense pathogens.

Unfortunately, researches on human NoVs have been hampered due to the fact that it cannot be grown in cell culture system and lack a robust small animal model for infectivity and pathogenesis study (35, 62). As a consequence, there are no vaccines or antiviral drugs are currently available for human NoV. Recent epidemiological studies found that severe clinical outcomes including death are often associated with high-risk populations such as the elderly, children, and immunocompromised individuals. From 1999-2007 human NoV caused, on average, 797 deaths per year in the USA. Mortality of NoV associated infection increases during the epidemic seasons and the burden of human NoV is much greater in the developing world. The CDC estimates that human NoV causes the death of 200,000 children under the age of 5 every year in developing countries. Therefore, there is an urgent need to develop an efficacious vaccine and therapeutic agent for control and prevent human NoV.

Antibody-based passive immunization has been shown to be an effective strategy to prevent and treat infectious diseases (61). Also, rapid and immediate protection can be achieved after passive immunization, for example, against agents of bioterrorism (18). Using mammalian serum to produce antibodies for oral administration has been described previously (27). However, its application has been limited by the high cost of large-scale antibody production and time consuming (65, 113). In addition, passive immunization with polyclonal antibodies has also been shown to have higher levels of protection compared to monoclonal antibodies (28). Immunoglobulin Y (IgY), the egg yolk antibodies generated as a passive immunity to embryos and baby chicks (83), can be a good alternative for large-scale production of polyclonal antibodies at a lower cost. Chicken IgYs are transferred from blood to the egg yolk during embryo development (113). IgY can be easily produced and purified with high yields from egg yolks of immunized hens through variable methods. In addition, production of antibodies via laying hens only requires the collection of eggs, and the animal number can be reduced due to the high and long-lasting titers produced in chickens (89). Therefore, the IgY technology is a safe, convenient, and inexpensive strategy to prevent and control infectious diseases, especially for gastrointestinal infections (3, 21).

Recently, it was reported that human NoV-specific IgY could be generated by immunization of hens with human NoV virus-like particles (VLPs), which were purified from insect cells using a baculovirus expression system. In addition, these IgYs can block the binding of human NoV VLPs to its receptors, the histo-blood group antigens (HBGA), suggesting that IgY may potentially be used as passive immunization and therapeutic agent for human NoV. Although these results are

promising, purification of VLPs from insect cells is time consuming and expensive which may limit the large-scale production of IgY. Previously, our laboratory developed a more efficient, convenient, and economical strategy to produce human NoV VLPs. Specifically, we constructed a recombinant vesicular stomatitis virus that expressing VP1 gene of human NoV (rVSV-VP1). It was found that VLP production by rVSV-VP1 was approximately10 times higher than that by insect cells-baculovirus expression system. As expected, vaccination of mice with rVSV-VP1 triggered significantly higher human NoV- specific humoral, cellular, and mucosal immunities than traditional VLP vaccination (76).

The objective of this study was to determine whether chicken yolk can be used a bioreactor to generate human NoV-specific IgY using rVSV-VP1 as an antigen. It was found that human NoV-specific IgY could be triggered in egg yolks from hens vaccinated with rVSV-VP1. It was also found that vaccination route affected the efficacy of IgY production in eggs. Specifically, hens vaccinated with rVSV-VP1 by intramuscular route produced 3 times more human NoV-specific IgY than by combination of intramuscular and nasal drop route. Importantly, human NoV-specific IgY produced by rVSV-VP1 vaccination was capable of blocking the binding of human NoV VLPs to type A, B, and O HBGA receptors. These data support the idea that recombinant rVSV-VP1 can be used as a highly effective antigen for large-scale production of human NoV-specific IgY for passive immunization and therapeutic agent.

2.3. Materials and methods

2.3.1. Virus and cell culture

Recombinant vesicular stomatitis virus expressing human NoV capsid protein (rVSV-VP1) was previously constructed in our laboratory (76)(Ma and Li, 2011). Working stocks of rVSV-VP1 were propagated in confluent BSRT7 cells (provided by Sean Whelan at Harvard Medical School, Boston, MA). Briefly, BSRT7 cells in T150 flask were infected by rVSV-VP1 at a multiplicity of infection (MOI) of 10. After 1 h of absorption, the inoculum was removed, and the cells were washed twice with Dulbecco's modified Eagle's medium (DMEM). After addition of 15 ml fresh DMEM (supplemented with 2% fetal bovine serum), the infected cells were incubated at 37°C in CO2 incubator. When extensive cytopathic effects (CPE) were observed, cell culture fluid was harvested, and virus titer was determined by plaque assay in Vero cells.

2.3.2. Multi-step growth curves in BSRT7 and chicken DF-1 cells

35 mm dishes were seeded with BSRT-7 or DF-1cells (kindly provided by Dr. Qingzhong Yu at USDA ARS, Athens, GA) and were infected with rVSV-VP1 at a multiplicity of infection (MOI) of 10. After 1 h of absorption, the inoculum was removed, the cells were washed twice with DMEM, 2 ml of fresh DMEM (supplemented with 2% fetal bovine serum) was added, and the infected cells were

incubated at 37°C. At the indicated intervals, 500 µl of aliquots of the cell culture fluid were removed and the same amount of fresh DMEM was added back to the virus-infected cells. Virus titers were determined by plaque assay in Vero cells.

2.3.3. Determination of the kinetics of VP1 expression in cell culture

6-well plates were seeded by BSRT7 or DF-1cells, and the cells were infected with rVSV-VP1 at an MOI of 10. At the indicated times, cells were lysed in lysis buffer containing 5% ß-mercaptoethanol, 0.01% NP-40, and 2% sodium dodecyl sulfate (SDS). Proteins were separated by 12% SDS-PAGE and transferred to a Hybond ECL nitrocellulose membrane (Amersham) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The blot was probed with guinea pig anti-human NoV VP1 antiserum (a generous gift from Dr. Xi Jiang) at a dilution of 1:6000, followed by horseradish peroxidase-conjugated goat anti-guinea pig IgG secondary antibody (Santa Cruz) at a dilution of 1:20,000. The blot was developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to Kodak BioMax MR film (Kodak).

2.3.4. Chickens and immunization

The animal study was conducted in strict accordance with USDA regulations and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and was approved by The Ohio State University

Institutional Animal Care and Use Committee (animal protocol no. 2013A00000011). Chickens were housed in cages inside high-security isolation rooms provided with HEPA-filtered intake and exhaust air at The Ohio Agriculture Research and Development Center, The Ohio State University. The animal care facilities at The Ohio State University are AAALAC accredited. Before animal study, blood samples were collected from each chicken to confirm that they were negative for human NoV antibody.

Six, 21-week-old, healthy White Leghorn chickens were provided by Dr. Lilburn, Department of Animal Sciences, The Ohio State University and were randomly divided into two groups (three chickens per group). Chickens in Group I were immunized intramuscularly by injecting 500 µl of DMEM containing 5×10^7 PFU of VSV-VP1 into three different locations of the pectoral muscle. Chickens in Group II were immunized by combination of intramuscular and intranasal routes. Specifically, 300 µl of VSV-VP1 (3×10^7PFU) was injected into three different locations of the pectoral muscle, and the remaining 200 µl of VSV-VP1 (2×10^7PFU) was used for nasal drop vaccination. At week 2 post-immunization, chickens in groups I and II were boosted with 5×10^7 PFU of VSV-VP1 via intramuscular and combination of intramuscular and intranasal routes, respectively. After immunization, eggs were collected daily until week 4 post-vaccination. Eggs that were collected one week before immunization were used as negative control. Eggs were stored at 4°C before IgY extraction.

2.3.5. Production and purification of human NoV VLPs by a baculovirus expression system

Purification of VLPs from insect cells was described previously with some minor modifications (1, 30, 53). *Spodoptera frugiperda* (Sf9) cells were infected with baculovirus expressing human NoV VP1 at an MOI of 10, and the infected Sf9 cells and cell culture supernatants were harvested at 6 days postinoculation. The VLPs were purified from cell culture supernatants and cell lysates by ultracentrifugation through a 40% (wt/ vol) sucrose cushion, followed by CsCl isopycnic gradient (0.39 g/cm^3) ultracentrifugation. Purified VLPs were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie blue staining. The protein concentrations of the VLPs were measured by using the Bradford reagent (Sigma Chemical Co., St. Louis, MO).

2.3.6. Extraction and purification of IgY from chicken egg yolks

IgY was extracted and purified from egg yolks using polyethylene glycol 8000 (PEG 8000, Sigma) precipitation method (Pauly et al., 2011) with some modifications. Briefly, egg yolks were diluted in three volumes of PBS (pH 7.4) and mixed, and PEG 8000 was added to a final concentration of 3.5%. After vortex, the mixture was continued to roll on a rolling mixer for 20 min., The mixtures were centrifuged at 13,000 g for 20 min at 4 °C, and the precipitated debris were removed. Subsequently, PEG 8000 was added to the supernatant to a final concentration of

8.5%, and the samples were mixed on a rolling mixer for 20 min. The mixtures were centrifuged again at 13,000 g for 20 min at 4 °C. The precipitated pellets containing IgY were dissolved in 10 ml of PBS and then precipitated again with 12% of PEG 8000 using the same procedures described above. The final pellets was dissolved in 2.0 ml of PBS, filtered through a 0.45 μm filter, and stored at -20 °C. The purity of the IgY was determined by SDS–PAGE followed by Coomassie blue staining.

2.3.7. Determination of human NoV-specific IgY and total IgY titers in egg yolk

Standard ELISA measured human NoV-specific IgY antibody titers. Briefly, 96-well microtiter plates were coated with 100μl of purified NoV VLP antigen (200ng/well) and incubated overnight at 4 °C. After blocking with 5% nonfat milk, 10 times serially diluted chicken IgYs were added to the antigen-coated wells and incubated at 37 °C for 1 h. After washing with PBST (PBS containing 0.05% Tween), goat anti-chicken IgY-HRP (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA) was added for 1 h. Plates were washed and developed with 75 μl of 3,3', 5,5'-tetramethylbenzidine (TMB), and the optical density (OD) at 450 nm was determined using an enzyme-linked immunosorbent assay (ELISA) plate reader. The IgYs from pre-immunized chicken yolks were used as controls.

To calculate the amount of total IgY and NoV-specific IgY, a standard curve was set up as follows: wells were coated with 100 μl of serially diluted pure chicken IgY (Promega, Madison, WI) at a concentration from 0.0075 μg/ml to 1 μg/ml. After washing with PBST, 100 μl of goat anti-chicken IgY-HRP (Santa Cruz

Biotechnology) at a dilution of 1:1000) were added and incubated at 37 °C for 1 h. The bound HRP was colorized by substrate reagent (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, Maryland, USA), followed by a reading of the signal intensity at 450 nm (Epoch Micro-Volume Spectrophotometer System, BioTek,Winooski, VT, United States). The resulting standard curve of absorbance was used to quantify the relative concentration of total IgY and NoV-specific IgY from the egg yolks by coating plates with either human NoV VLP or rabbit anti-chicken IgY antibodies (10μg/ml, Sigma) to capture the total IgY or NoV-specific IgY.

2.3.8. Analysis of chicken IgY by SDS-PAGE

The purified IgYs from egg yolks were analyzed by SDS-PAGE. Samples were boiled for 5 min in loading buffer containing 1% SDS, 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl (pH 6.8), and 5% glycerol and loaded onto a 12% polyacrylamide gel. Proteins were visualized by Coomassie blue staining.

2.3.9. Characterization of IgY by Western blot analysis

Specific reactions of the chicken IgY with NoV capsid protein were examined by Western-blot analysis. The purified human NoV virus-like particles (VLPs) were separated by conventional 12% SDS-PAGE and transferred to a Hybond ECL

nitrocellulose membrane (Amersham) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). After blocking with 5% nonfat milk, the membrane was incubated with anti-NoV-specific IgY or non-specific IgY (1:1000) in 1% nonfat milk-PBS at 4 °C overnight, followed by horseradish peroxidase (HRP)-conjugated goat anti-chicken IgY secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:5000. The blot was developed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and exposed to Kodak BioMax MR film (Kodak).

2.3.10. HBGA binding assay

The saliva-based HBGA binding and blocking assays were performed as described previously (28, 36, 51). To avoid potential NoV-specific antibodies in the saliva that may interfere with the receptor-binding assay, saliva samples were boiled before being used in the assays. The boiled human saliva samples with known HBGA phenotypes (A, B, or O) were diluted 1000-fold and coated on 96-well microtiter plates at 4 °C overnight. After blocking with 5% nonfat milk in PBS, human NoV VLPs were added to a final concentration of 4μg/ml. The bound VLPs were detected by using serially diluted IgYs (from 1:1,000 to 1:128,000), followed by the addition of HRP-conjugated goat anti-chicken IgY at a dilution of 1:5000. The color was then developed by adding tetramethylbenzidine peroxidase liquid substrate (Kirkegaard and Perry Laboratory) and stopped after 10 min of incubation at 22°C by adding 1 mol/L sulfuric acid. Optical density (OD) was measured at 450 nm with the use of a Epoch Micro-Volume Spectrophotometer System (BioTek).

2.3.11. HBGA blocking assay

HBGA blocking assay was performed to determine the inhibitory activity of IgY against the binding of human NoV VLPs to the HBGA antigens. The boiled human saliva samples with known HBGA phenotypes (A, B, or O) were diluted 1000-fold and coated on 96-well microtiter plates at 4 °C overnight. The human NoV VLPs were pre-incubated with serially diluted IgYs for 1 h at 37°C, and IgY-VLP solutions were added to the saliva-coated wells. Plates were washed 3 times with 0.1 mol/L sodium phosphate buffer (pH, 6.4). Then, a guinea pig anti-human NoV VLP antiserum at a dilution of 1:1000 was added and incubated for 1 h at 4°C. The plates were washed again, and HRP-conjugated goat anti-guinea pig IgG (at dilution of 1:5000) was added and incubated for 1 h at 4°C. The color was then developed by adding tetramethylbenzidine peroxidase liquid substrate (Kirkegaard and Perry Laboratory) and stopped after 10 min of incubation at 22°C by adding 1 mol/L sulfuric acid. The blocking rates were calculated by comparing the optical densities (ODs) measured with and without blocking by the chicken IgYs. The IgYs from chickens before immunization were used as controls (51). Blank wells were incubated with buffer instead of IgY-VLP and served as negative control whereas VLP binding to carbohydrates in the absence of IgY sample was used as a positive control.

2.3.12. The effects of pH on the stability of human NoV-specific IgY

For the pH stability, the purified total IgY solution (1 ml, 1:100, pH 7.4) was diluted in 0.1mol/L sodium phosphate buffer (pH 6.4). The pH of the solution was adjusted using either HCl or NaOH to a final pH ranging from 2 to 11. The solution was incubated at 37 ^{o}C for 3 h, followed by neutralization by adding 5 X PBS (pH =6.4) to a final pH of 7. The HBGA blocking assays were performed to measure the activity of IgY as described above.

2.3.13. The effects of heat treatment on the stability of human NoV-specific IgY

To determine heat stability of human NoV-specific IgY, the purified total IgY solution (1 ml, 1:100, pH 7.4) was treated at temperature ranging from 4 to 80 ^{o}C for up to 30 min. After heat treatment, the samples were cooled quickly on ice. The HBGA blocking assays were performed to measure the activity of IgY as described above.

2.3.14. Statistical analysis

Quantitative analysis was performed by either densitometric scanning of autoradiographs or by using a Typhoon PhosphorImager and ImageQuant TL software (GE Healthcare, Piscataway, NJ). Each experiment was performed three to six times. Statistical analysis was performed by one-way multiple comparisons using

SPSS software 13.0 (SPSS, Chicago, IL). A *P* value of <0.05 was considered statistically significant.

2.4. Results

2.4.1. Comparison of the growth kinetics and VP1 expression of rVSV-VP1 in BSRT7 and DF-1 cell

Previously, it was shown that recombinant rVSV-VP1 replicated to a high titer in BSRT7 cells, a mammalian cell line (Ma and Li, 2011). We first aimed to determine whether rVSV-VP1 replicated efficiently in DF-1 cells derived from chicken embryo. Briefly, BSRT7 and DF-1 cells were infected with rVSV-VP1 at an MOI of 10 and the kinetics of viral replication was determined at time points from 0-48 h post-infection. As shown in Fig. 14A, rVSV-VP1 replicated efficiently in both DF-1 and BSRT7 cells. Viral titer gradually increased at 2 h post-infection and reached a peak titer of 7.0×10^9 pfu/ml at approximately 24 h post-infection. However, in BSRT7 cell, the virus titer started to increase at 4 h post-infection, and reached a peak titer of 9.6×10^9 at approximately 30 h post-infection. In addition, virus titer in DF-1 cell decreased after 24 h post-infection because of the cell death. Overall, there is no significant difference in viral replication in DF-1 and BSRT7 cells ($P<0.05$).

To further examine the expression of human NoV VP1 protein by VSV vector in two different cell lines, we monitored the kinetics of VP1 expression in BSRT7 and DF-1 cells. Briefly, BSRT7 and DF-1 cells were infected with rVSV-VP1 at an MOI of 10 and cell lysates were harvested at the indicated times. The expression of VP1 was determined by Western blot. The VP1 expression was detectable at 4 h post-

infection in both cell lines, and reached a peak during 10-12 h post-infection (Fig.14 B, C). There was no significant difference ($P<0.05$) in VP1 protein expression in these two cell lines during 4-24 h post-infection. However, VP1 expression in DF-1 decreased after 30 h post-infection that was probably due to the cell death.

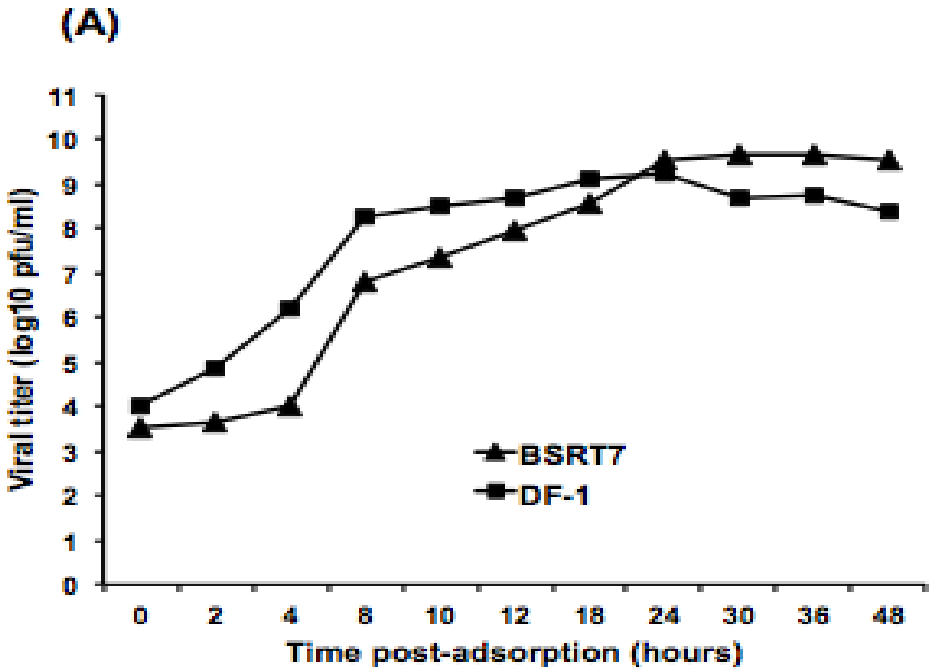
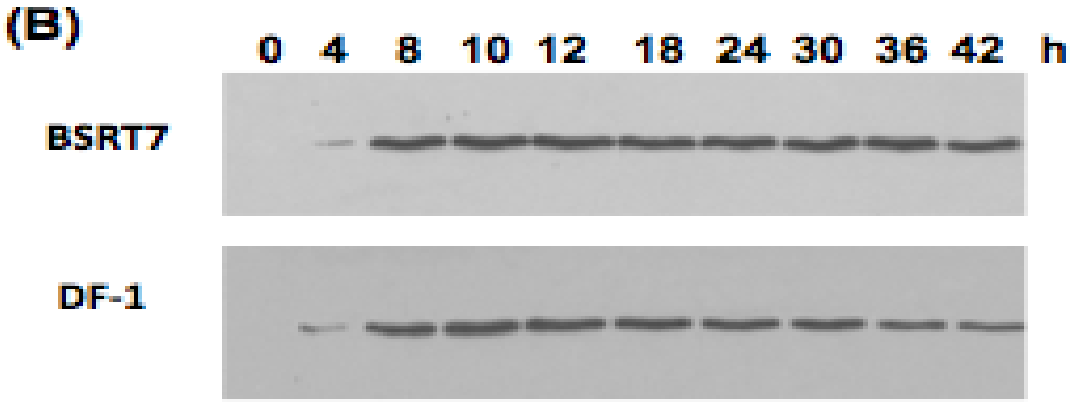
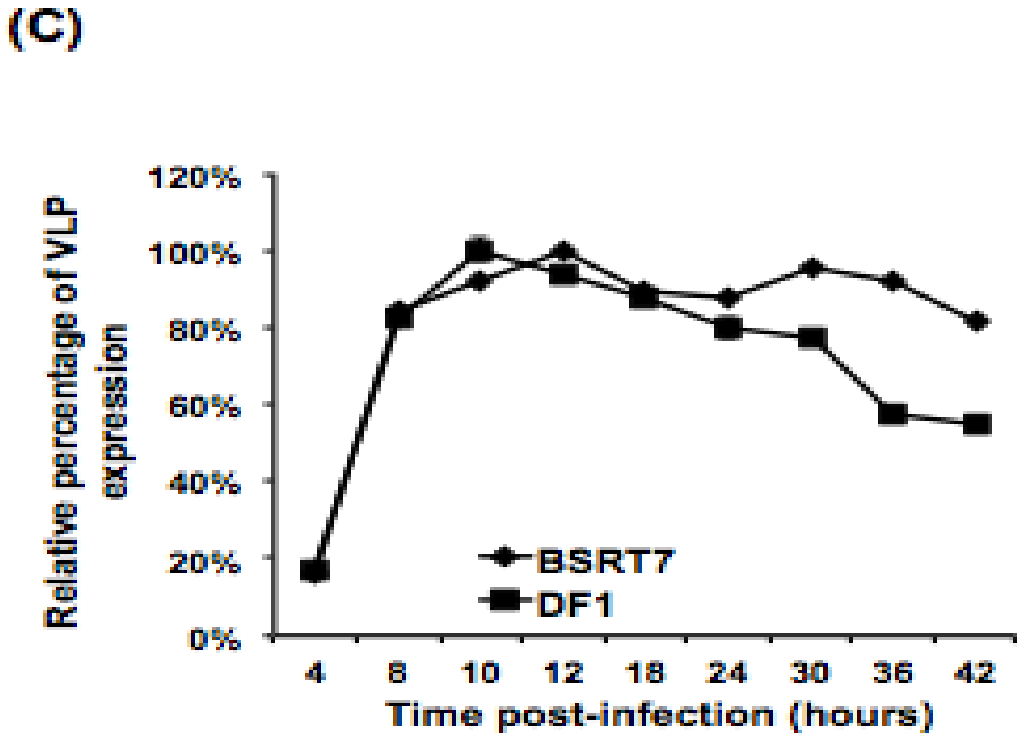


Figure 14. The growth kinetics and VLP expression of VSV-VP1 in BSRT7 and DF-1 cell. (A) Confluent BSRT7 and DF-1 cells were infected with individual viruses at an MOI of 10. After 1 h incubation, the inoculum was removed, the cells were washed with DMEM, and fresh medium (containing 2% fetal bovine serum) was added, followed by incubation at 37°C. Samples of supernatant were harvested at the indicated intervals over a 48-h time period, and the virus titer was determined by plaque assay. (B) Dynamics of VP1 expression in cell lysate by Western blot. BSRT-7 and DF-1 cells were infected with rVSV-VP1 at an MOI of 10. Cytoplasmic extracts were harvested at indicated time points. Equal amounts of total cytoplasmic lysate were analyzed by SDS-PAGE, followed by Western blot analysis using guinea pig anti-human NoV VP1 antiserum. (C) Quantitative analysis of VP1 expression in cytoplasmic lysate. Three independent experiments were used to generate the quantitative analysis shown. Data was expressed as the mean ± the standard deviation. (Continued)

Figure 14. Continued





2.4.2. Biosafety of rVSV-VP1in chickens

Previously, Ma and Li (2011) showed that recombinant rVSV-VP1 was not only attenuated in vitro and in vivo, but also triggered a high level of mucosal, humoral, and cellular immunities in a mouse model. This suggests that rVSV-VP1 can be used a robust bioreactor for large-scale production of human NoV-specific antibody for therapeutic and diagnostic purpose. To test this hypothesis, we determined whether rVSV-VP1 could stimulate human NoV-specific IgY in chickens. To do this, rVSV-VP1 was inoculated into chickens by two routes: intramuscular and combination of intramuscular and intranasal route. After vaccination, the safety of rVSV-VP1 in chickens was monitored daily. No abnormal reaction was observed in chickens vaccinated by both routes. rVSV-VP1 vaccination did not affect feed intake, weight gain, and egg production. This result suggests that rVSV-VP1 was safe to chickens.

2.4.3. Purification and characterization of human NoV-specific IgY from chicken yolks

After rVSV-VP1 vaccination, eggs from each hen were collected daily. To determine whether rVSV-VP1 triggers human NoV-specific IgY, total IgY was purified from each egg collectively at weeks 1, 2, 3, 4 post-vaccination using the protocol described in Materials and Methods. To examine the purity of total IgY, 5 μl of total IgY from one egg collectived at week 4 post-vaccination was analyzed by

SDS-PAGE. As shown in Fig. 15 A, two protein bands with molecular weight of 68 and 27 kDa were observed, which were consistent with the size of heavy and light chain of IgY, respectively. SDS-PAGE also revealed a protein band approximately 38-40 kDa, which may be α-2-glycoprotein according to previous reports.

To determine whether purified total IgYs contain human NoV-specific IgY, Western blot was performed using human NoV VLPs purified from insect cells by a baculovirus expression system. Briefly, human NoV VLPs were separated by SDS-PAGE, followed by Western blotting, which used total IgY as a polyclonal antibody against VP1 protein. We first showed that human NoV VLPs were detected by serum IgG (Fig. 15C). As shown in Fig. 15E, the molecular weight of purified VLPs was approximately 56kDa in SDS-PAGE, which corresponds to the size of human NoV VP1 protein. After Western blot, a strong protein band corresponding to VP1 protein was visualized, demonstrating that VLPs reacted with human NoV-specific IgY in the total IgY. As a control, no protein bands were identified in Western blot using total IgY from eggs prior to rVSV-VP1 vaccination (Fig.15D). Therefore, these results confirmed that rVSV-VP1 vaccination triggered human NoV-specific IgY in egg yolks.

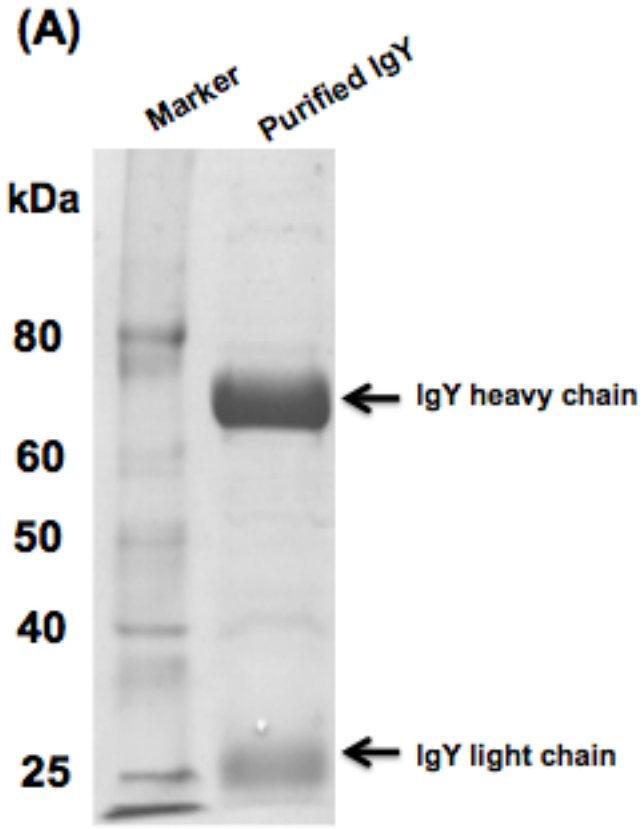
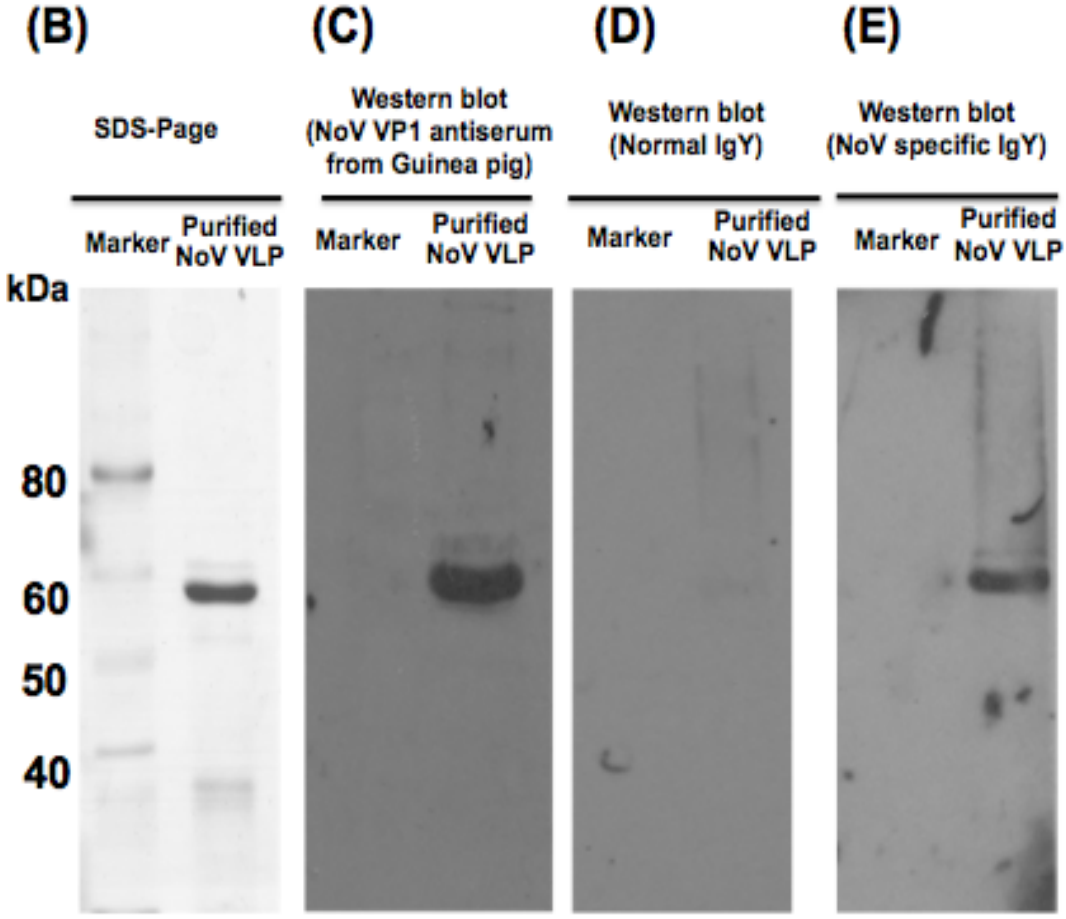


Figure 15. Characterization of total IgY and human NoV-specific IgY in egg yolks. (A) Analysis of total IgY by SDS-PAGE. (B) SDS-PAGE analysis of purified human NoV VLPs. (C) Western blot analysis of human NoV VLPs using guinea pig anti-HuNoV VP1. (D) Western blots analysis of human NoV VLPs using total IgY purified from eggs collected before rVSV-VP1 vaccination (non-specific IgY). (E) Western blots analysis of human NoV VLPs using IgY purified from eggs collected at week 4 post-vaccination (specific IgY). (Continued)

Figure 15. Continued



2.4.4. Kinetics of human NoV specific IgY responses in chicken egg yolks after rVSV-VP1 vaccination

To determine the kinetics of human NoV-specific IgY responses, ELISA using a commercially available IgY as a standard measured the amount of total IgY and human NoV-specific IgY. Briefly, 96-well plates were coated with 200 ng of purified

human NoV VLP antigen in each well and were reacted with serially diluted chicken IgY at 37 °C for 1 h. After reacting with HRP labeled goat anti-chicken IgY followed by addition of substrate reagent, an ELISA reader read the OD value at 450 nm. Subsequently, the amount of total IgY and human NoV-specific IgY was quantified using standard curve generated by commercially available IgY. As shown in Fig. 16A, the amount of human NoV-specific IgY gradually increased after rVSV-VP1 immunization. Interestingly, at weeks 2, 3, and 4 post-vaccination, the levels of human NoV-specific IgY in intramuscular vaccination group were significantly higher than those in the combined intramuscular and nasal drop vaccination group ($P<0.05$). At week 4 post-vaccination, NoV-specific IgY in intramuscular group reached 4.8 mg/yolk whereas only 1.8 mg/yolk NoV-specific IgY was detected in intramuscular and nasal drop group.

Using a similar method, the amount of total IgY in each egg yolk was determined. As shown in Fig.16B, there was no significant difference in total IgY between the two vaccination groups ($P<0.05$). In addition, there was no significant difference in total IgY from eggs collected before and after rVSV-VP1 vaccination ($P<0.05$). Under our experimental condition, the amount of total IgY in each yolk ranged from 84.4 ± 3.8 to 86.4 ± 7.8 mg.

Next, we calculated the percentage of human NoV-specific IgY in total IgY in each egg yolk. As shown in Fig.16C, the percentage of human NoV-specific IgA gradually increased from 0.7% to 5% in the intramuscular immunization group. In contrast, the percentage of human NoV-specific IgA ranged from 0.05% to 2% in the combined immunization group. Collectively, these results suggest that both immunization routes were capable of producing human NoV-specific IgY, and

intramuscular injection alone was more effective in triggering NoV-specificIgY than combination of intramuscular and nasal drop route.

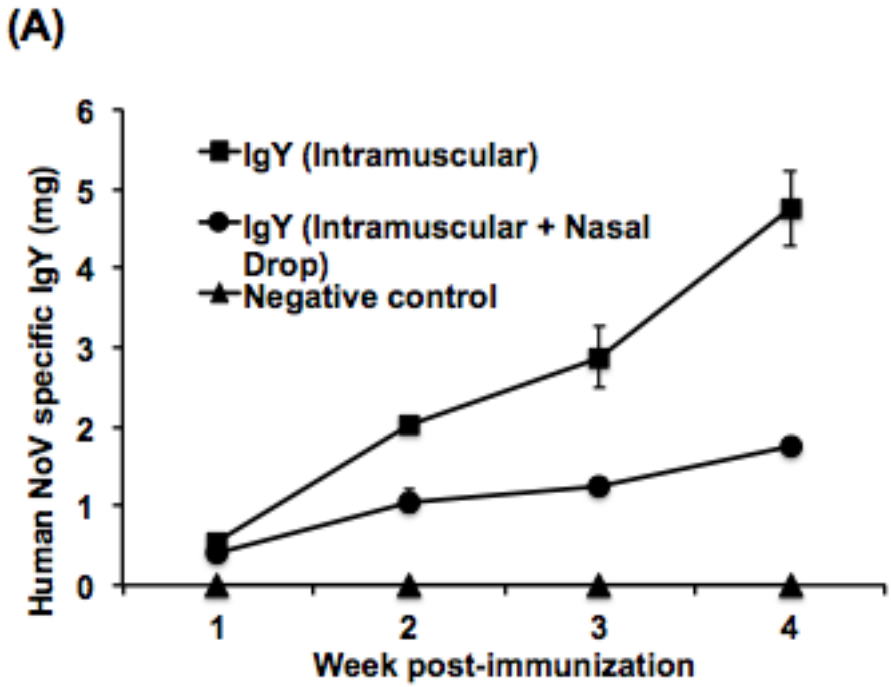
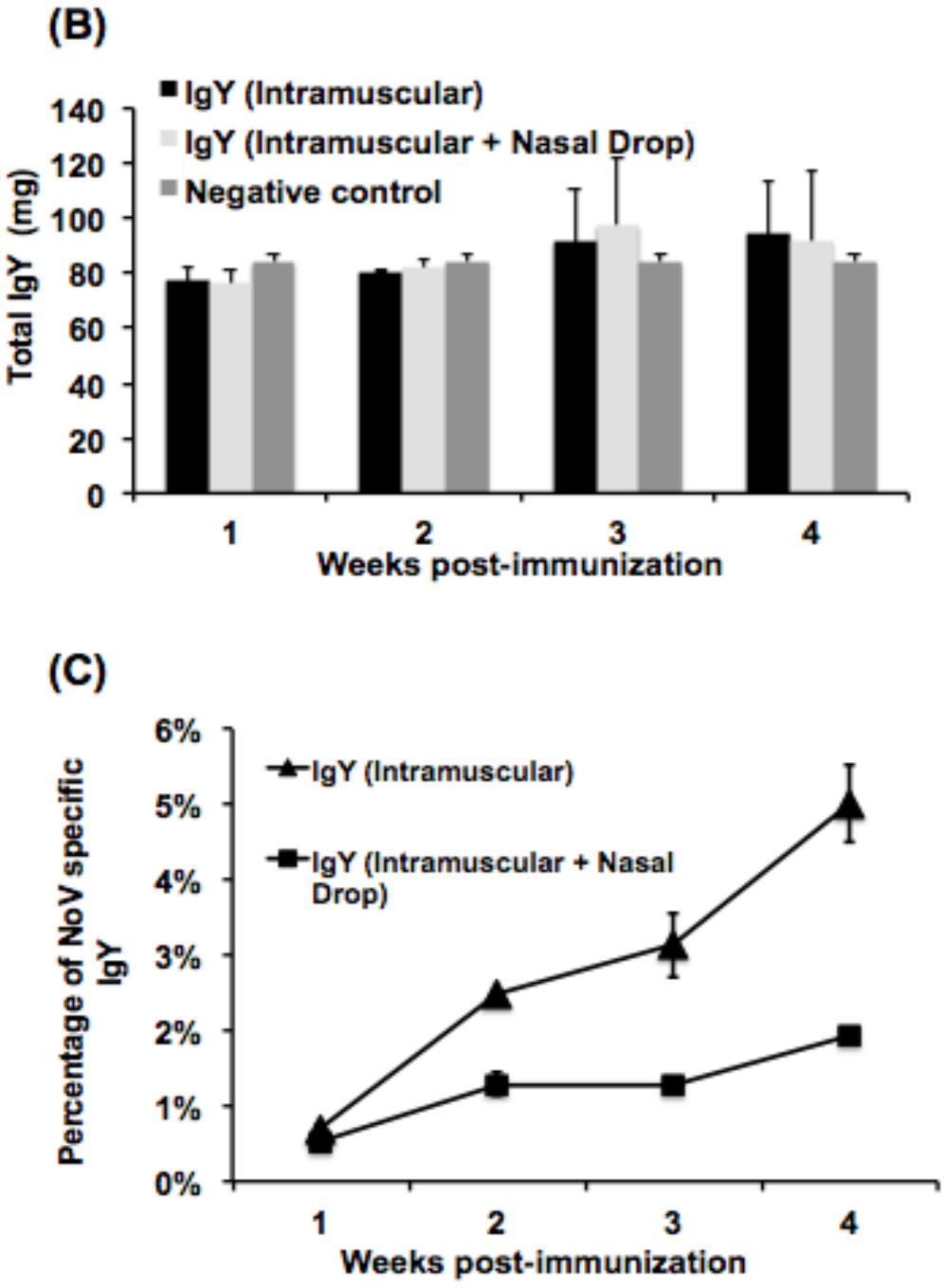


Figure 16. Kinetics of IgY production in chicken vaccinated with recombinant rVSV-VP1. (A): The amount of human NoV-specific IgY in egg yolks. (B) The amount of total IgY in egg yolks. (C) The percentage of human NoV-specific IgY in total IgY. (Continued)

Figure 16. (Continued)



2.4.5. Development of a saliva-based HBGA binding assay using purified IgY as a detection antibody.

All viruses must bind to cellular receptor(s) to initiate an infection. Human NoV utilizes HBGAs as functional receptors. Human NoV VLPs possess authentic receptor binding activity that can be detected by HBGA binding assay. In this assay, human saliva containing HBGA receptors (types A, B or O) or synthetic HBGAs can be recognized by human NoV VLPs, which can be further detected by anti-human NoV serum and a HRP-conjugated second antibody. Therefore, we determined whether total IgY derived from rVSV-VP1 vaccinated hens could be used as the primary antibody for the HBGA binding assay. Briefly, 96-well plates were coated with type A, B, or O human saliva, and 200 ng of human NoV VLPs were added to bind the HBGA receptor. After 1 h incubation, a serial dilution of total IgY was added followed by addition of HRP-conjugated goat anti-chicken IgY. After the addition of substrate reagent, an ELISA reader measured OD450. As expected, purified total IgY from rVSV-VP1 vaccinated groups strongly reacted with NoV VLPs in saliva-based HBGA binding assays (Fig. 17A). In contrast, control IgY from pre-immunized hens was negative in this assay. Overall, total IgY from intramuscular vaccination group had a significant stronger binding affinity than that in combined vaccination group ($P<0.05$). In addition, it appears that total IgY had a stronger binding activity to type A saliva compared to types B and O saliva. These results suggest that purified total IgY induced by rVSV-VP1 vaccination could be used as a primary antibody to measure binding of human NoV VLP to all three types of saliva in HBGA binding assays.

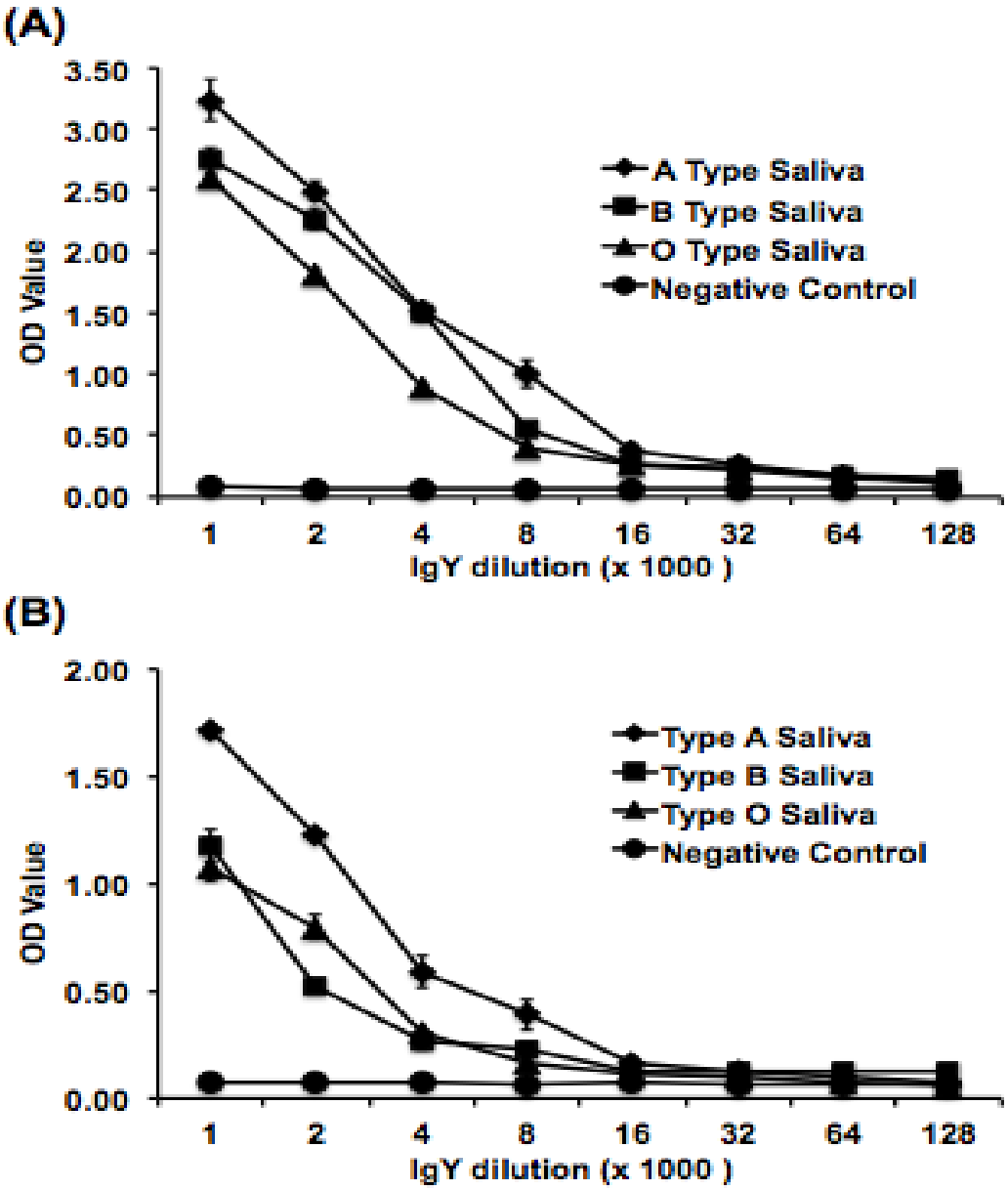


Figure 17. Histo Blood Group Antigen (HBGA) binding assay using purified total IgY as primary antibody. (A) Saliva-based HBGA binding assays using IgYs isolated from intramuscular vaccinated hens. (B) Saliva-based HBGA binding assays using IgYs isolated from intramuscular and nasal drop vaccinated hens. Each data point represents an average value of binding assays using IgYs isolated from three eggs.

2.4.6. IgY antibodies blocked the binding of human NoV VLPs to the HBGA receptors

Next, we determined whether the purified IgY has potential antiviral activity that can be developed as a therapeutic agent for human NoV infection. Since human NoV cannot be grown in cell culture, there is no standard virus-neutralizing assay. Thus, it is not feasible to directly measure the antiviral activity of IgY using cell culture-based assay. Recently, a HBGA blocking assay, which measures the ability of antibody blocks the binding of human NoV VLPs to the HBGA receptors, has been reported. Presumably, blockage of viral receptor binding activity will inhibit viral attachment, entry, and subsequent viral infection. Thus, this assay can serve as a useful surrogate assay for serum-virus neutralization assay.

To measure the inhibitory activity of IgY against the binding of human NoV VLPs to the HBGA receptors, we performed a saliva-based HBGA blocking assay. Briefly, similar to HBGA binding assay, 96-well plates were coated with a known saliva type (A, B or O) at 4°C overnight. The VLPs were pre-incubated with the serially diluted IgY at 37°C for 1 h before adding to the saliva-coated wells. Then, a guinea pig anti-NoV VLP antiserum was added, followed by the addition of HRP-conjugated goat anti-guinea pig IgG. After the addition of substrate reagent, OD_{450} was measured by an ELISA reader. Percent of blocking activity was calculated by comparing the OD values measured with or without blocking by the chicken IgYs. As shown in Fig .18, total IgY antibodies isolated from egg yolks of rVSV-VP1 vaccinated groups were capable of blocking the binding of NoV VLP to HBGA receptors (A, B, or O antigen) in a dose-dependent manner with a BT50 (a serum

dilution with 50% blocking activity) of about 1:400, 1: 800 and 1: 100, respectively. As controls, IgY purified from egg yolks of chickens before immunization did not have detectable blocking activity. Again, total IgY from intramuscular group had a significantly higher blocking activity compared to the intramuscular and nasal drop group ($P<0.05$). Therefore, it was concluded that IgY from rVSV-VP1 vaccinated hens specifically blocked the binding of human NoV VLPs to the HBGA receptors.

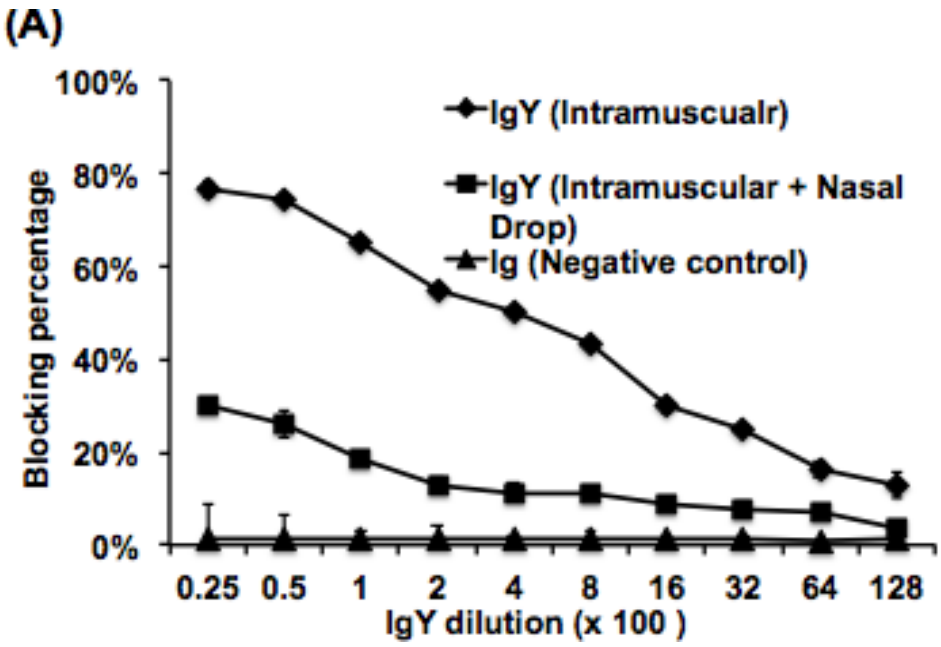
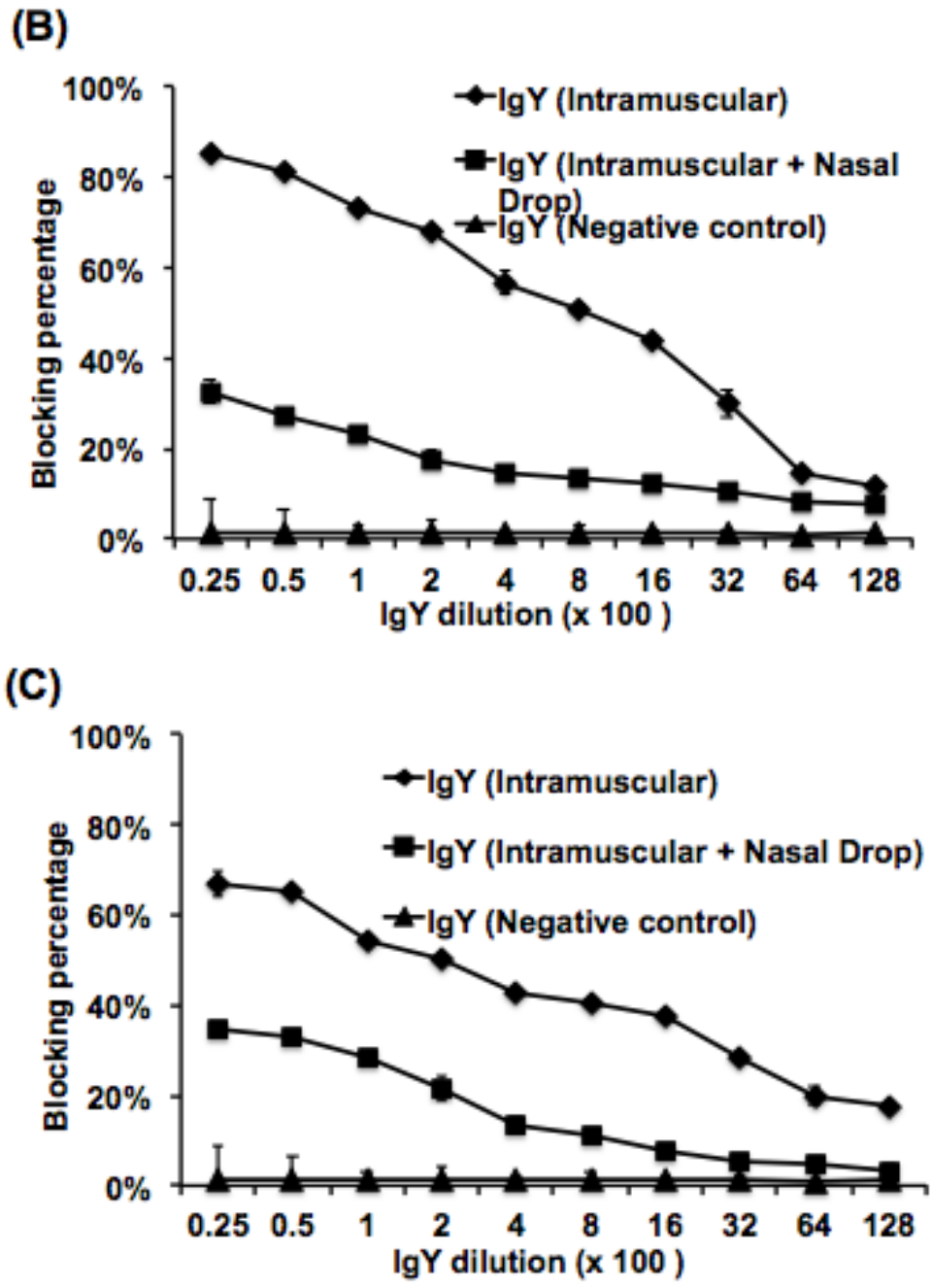


Figure 18. Chicken IgY induced by rVSV-VP1 vaccination blocked the binding of human NoV VLPs to HBGA receptors. (A) IgY blocked binding of human NoV VLPs to type A antigen receptors. (B) IgY blocked binding of human NoV VLPs to type B antigen receptors. (C) IgY blocked binding of human NoV VLPs to type O antigen receptors. (Continued)

Figure 18. (Continued)



2.4.7. Thermal stability of human NoV-specific IgY

To investigate thermal stability of IgY, IgY solution was incubated at temperature ranging from 4 to 80◦C for up to 30 min, and the receptor blocking activity was measured by saliva-based HBGA blocking. As shown in Fig. 19 A, B, and C, human NoV-specific IgYs retained wildtype level of blocking ability to all three types of saliva at the temperatures below 70 ◦C. However, the blocking activity significantly impaired at the temperatures above 75 ^{o}C ($P<0.05$).

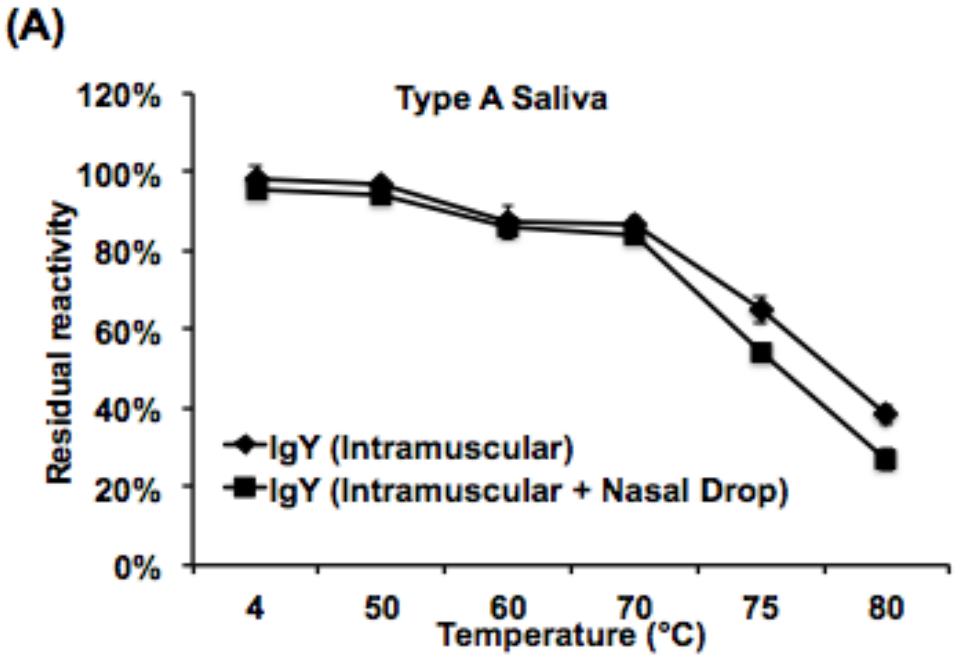
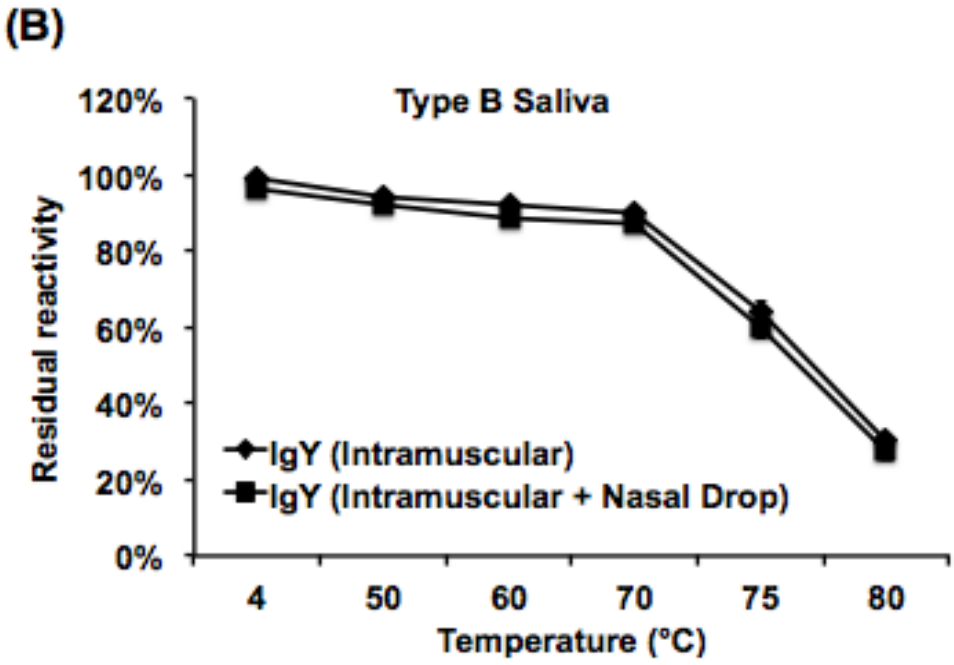
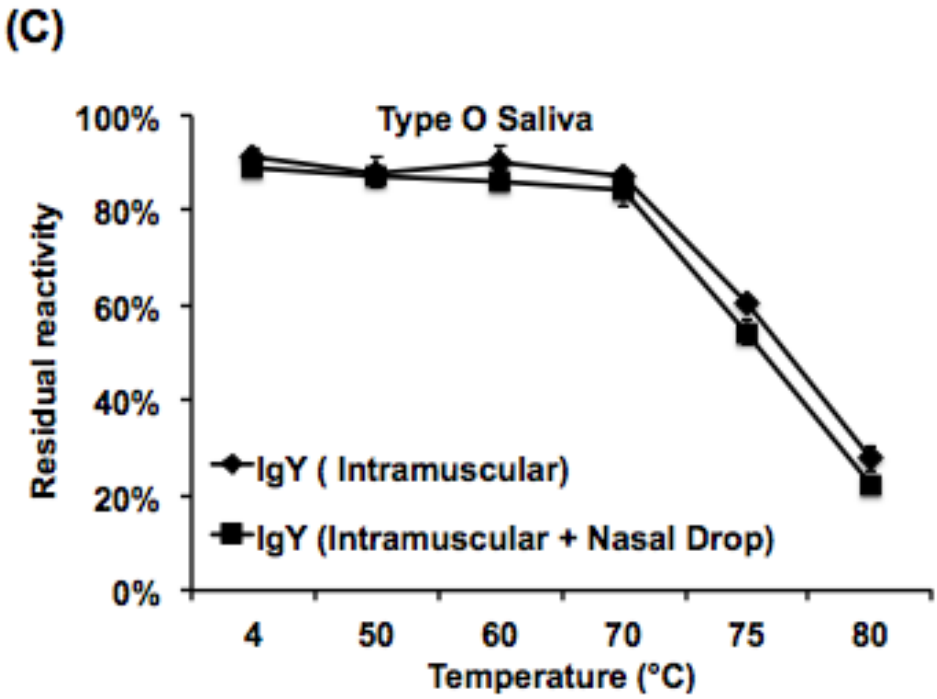


Figure 19. Effects of temperature on the ability of IgY to block the binding of NoV VLPs to HBGAs. (A) Blockage activity to type A saliva. (B) Blockage activity to type B saliva. (C) Blockage activity to type O saliva. A percentage (%) of residual blocking activity in comparison with an untreated sample is shown. Each data point represents the average of three replicates. (Continued)

Figure 19. (Continued)





2.4.8. pH stability of human NoV-specific IgY.

Next, we determined whether human NoV-specific IgY is stable in acid and alkaline environments. To do this, purified IgY was diluted 100 times in PBS solution, and the pH of the solutions was adjusted with either HCl or NaOH to a final pH of 2–11. After incubation at 37 ◦ C for 3 h, the solution was adjusted to neutral pH, and the receptor blocking activity was measured by HBGA blocking assay. As shown in Fig. 20 A, B, and C, the blocking activity of the chicken IgY remained stable at pH 4–9 for 3 h. However, a significant decrease of blocking activity was observed when the pH is lower than 4 or higher than 9.

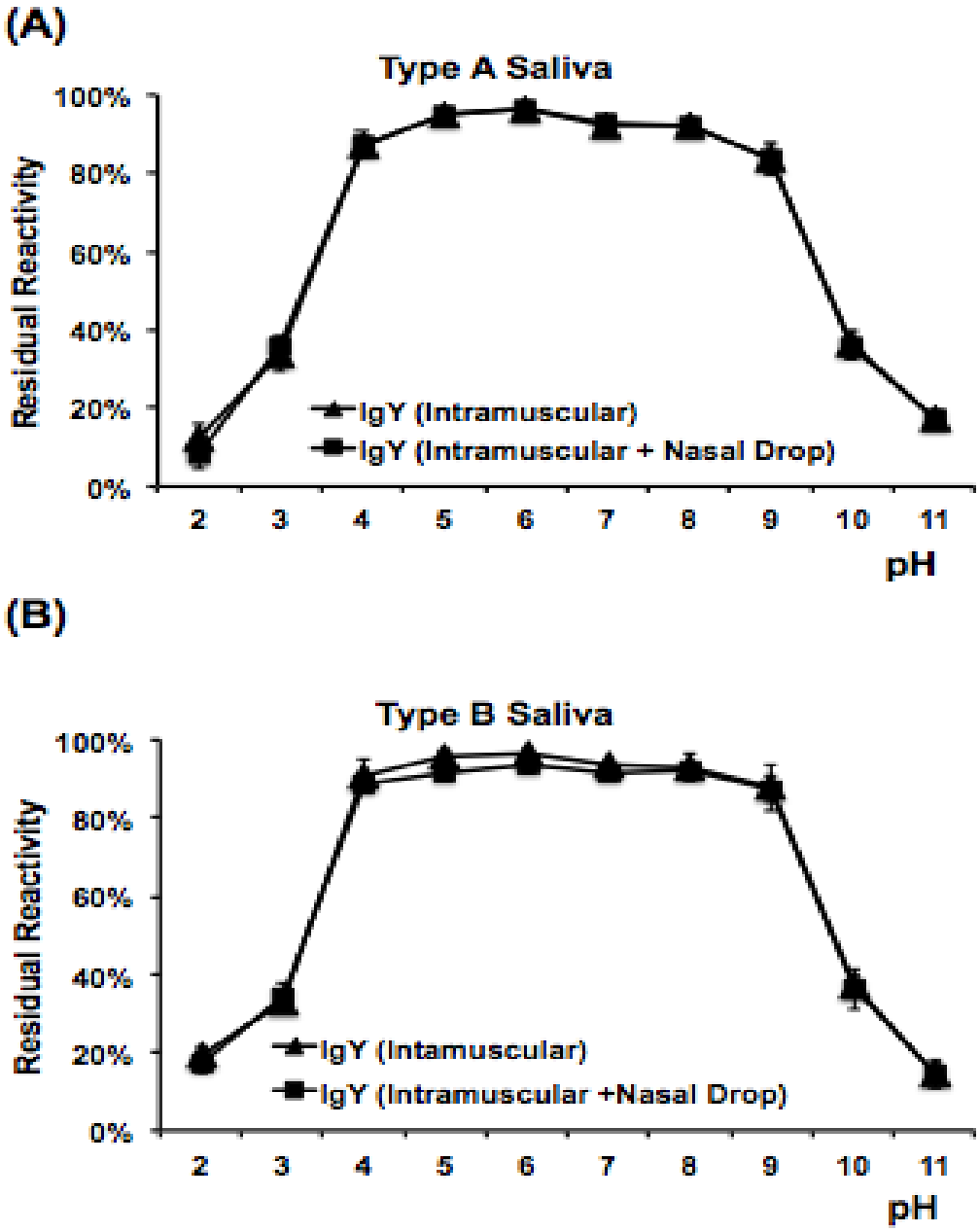
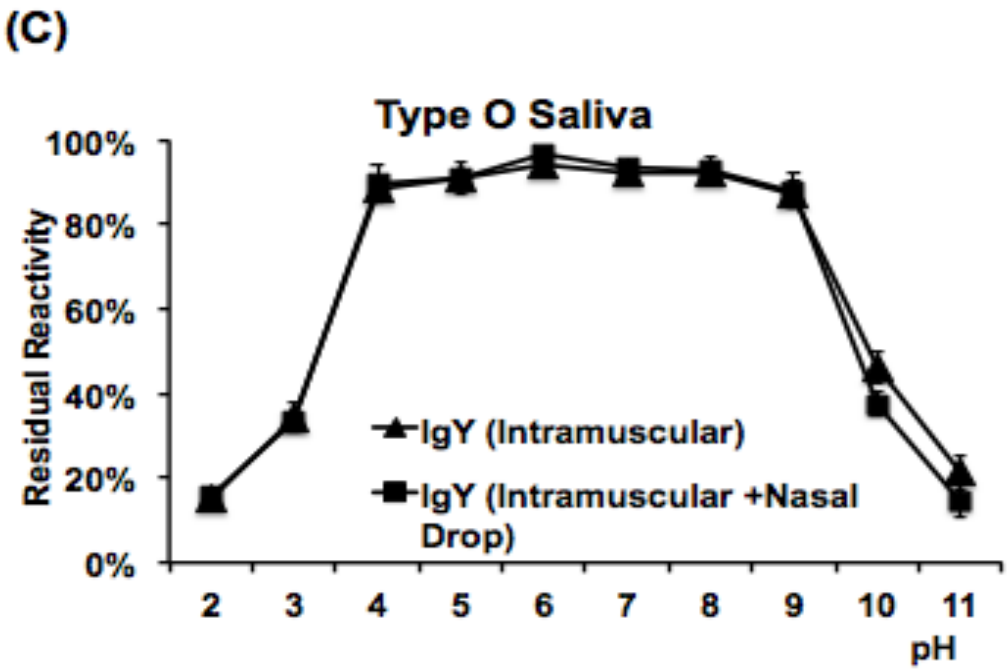


Figure 20. Effects of pH on the ability of IgY to block the binding of NoV VLPs to HBGAs. (A) Blockage activity to type A saliva. (B) Blockage activity to type B saliva. (C) Blockage activity to type O saliva. A percentage (%) of residual blocking activity in comparison with an untreated sample is shown. Each data point represents the average of three replicates. (Continued)

Figure 20. (Continued)



2.5 Discussion

In this study, we produced human NoV-specific IgY in egg yolks in white leghorn chickens immunized with recombinant rVSV-VP1. We found that intramuscular immunization alone was more efficient in triggering NoV-specific IgY compared to the combination of intramuscular and nasal drop immunization.We demonstrated that these specific antibodies strongly reacted with human NoV VLPs by ELISA and Western blot, and were capable of blocking human NoV-HBGA receptor interactions.

VSV-based vaccine for production of human norovirus-specific IgY in chickens. The natural hosts of VSV are cattle, horse, deer, and pig. However, VSV

has a board tissue tropism and can replicate to a high titer in many mammalian cell lines, insect cell, yeast, worm, and C elegant. In this study, we found that VSV-VP1 replicated to a high titer in DF-1 cells, a continuous cell line derived from chicken embryo fibroblasts. The virus yields in DF-1 cells were comparable to those produced in BSRT7 cell, a mammalian cell line. In addition, rVSV-VP1 produced similar amounts of human NoV VLPs in both DF-1 and BSRT7 cells, suggesting that VP1 can be highly expressed in chicken cell lines. However, replication capability of VSV in avian species in vivo is poorly understood. The only report of VSV infection in chickens came from the study of VSV-based influenza virus vaccine. It was found that chickens vaccinated with VSV expressing the HA antigen of highly pathogenic avian influenza virus (H7N1) triggered a high level of serum neutralizing antibody and provided complete protection against lethal challenge of avian influenza H7N1 (58). In this study, we found that hens vaccinated with rVSV-VP1 triggered a high level of human NoV-specific IgY in yolks, further supporting that chickens were susceptible to VSV infection. Immunization route is one of the major factors that can affect IgY production depending on the type of antigens. In this study, we found that intramuscular vaccination triggered a higher human NoV-specific IgY than combination of intramuscular and nasal drop vaccination although the total IgY levels were similar between two vaccination routes. Our original rationale to include nasal drop vaccination is that it may trigger a higher mucosal immunity since egg yolk is developed in reproductive tract of chickens. In fact, our previous study in mice showed that oral and intranasal vaccination of rVSV-VP1 trigged a high level of both serum and mucosal antibody(76). Unexpectedly, intramuscular vaccination alone was better than combined vaccination for yolk IgY production.

One of the major concerns of VSV-based gene delivery is the safety, particularly since VSV is neurotropic. VSV infection in ruminant animal causes vesicular lesions in the mouth, teats, and feet. In mice, wild type VSV can cause acute brain infection and fatal encephalitis. However, insertion of human NoV VP1 into VSV vector significantly attenuated the virus in a mouse model. In this study, rVSV-VP1 did not cause any abnormal reactions or growth characteristics in chickens, further proved that rVSV-VP1 was attenuated in vivo.

Although a recent report showed that human NoV-specific IgY can be isolated in hen vaccinated with purified VLPs from insect cells using baculovirus expression system(28). The level and duration of IgY production is limited because VLPs are non-replicating protein antigens. There are many advantages of using live attenuated rVSV-VP1 for IgY production. First, rVSV-VP1 grows to a high titer in a wide range of cell lines including chicken cells. Second, replication of rVSV-VP1 in chickens resulted in synthesis of large amount of VLPs that in turn triggered a high level of antibody. Third, it is more economical, and time-saving approach. It does not require purification of VLPs, which is expensive. Thus, rVSV-VP1 is an ideal live attenuated vaccine for large-scale production of IgY in chickens.

Human NoV-specific IgY as potential passive immunization and therapeutic agent for human NoV and other enteric viruses. Human NoV is a leading cause of viral gastroenteritis worldwide. Despite significant social, health, and economical burden it causes, no FDA approved vaccine or therapeutic strategy available. Epidemiology studies showed that human NoV could cause lethal infection in humans, particularly in high-risk populations, such as infants, young children, the

elderly, and immunocompromised individuals. Thus, development of a safe and effective therapy strategy is urgently needed.

In this study, we found that human NoV specific IgYs isolated from egg yolks were biologically functional in vitro. First, human NoV specific IgYs can react with VLPs in ELISA and Western blot. Second, similar to serum IgG, human NoV specific IgYs can be used as a primary antibody in HBGA binding assay. Third, human NoV-specific IgYs, but not control IgY, were able to block the binding of NoV VLPs to types A, B, and O HBGAs in human saliva in a HBGA blocking assay. Because of uncultivability of human NoV, it is not known whether human NoV specific IgY can directly neutralize the infectious human NoV. However, blockage of virus-receptor interaction will likely block the infectivity of human NoV, which will further prevent human NoV infection and illness. In 2010, Reeck et al. found a direct correlation between the ability of an antibody to block VLP-HBGA binding and protection against NoV infection and illness in a NoV human challenge study. In addition, Nurminen et al., (2011) showed that children could be protected from a GII.4 NoV infection due to the pre-existing HBGA blocking antibodies. Thus, the IgYs developed in this study can likely be used in a passive immunization approach to prevent and treat NoV infection and illness.

The concept of IgY passive immunization has been well developed in rotavirus, another important enteric virus, and in vivo animal models. It was found that passive immunization of IgY could protect neonatal calves from bovine rotavirus -induced diarrhea (67). In a mouse model, it was found that IgY could prevent murine rotavirus infection (46), bovine rotavirus-induced diarrhea (66), and human rotavirus-induced gastroenteritis (33). Recently, human rotavirus-specific IgY administered

orally as a milk supplement passively protects neonatal pigs against an enteric human rotavirus infection. Porcine epidemic diarrhea virus (PEDV) caused severe economic losses in Europe, Asian, and recently US. PEDV infection in piglets developed severe diarrhea, vomiting, fecal shedding, viremia, and severe atrophic enteritis. Kweon et al. (2000) found that IgY passive immunization can protect piglets against PEDV infection. Recently, others and we developed a gnotobiotic pig model for human NoV infection. It will be interesting to investigate whether human NoV-specific IgY can protect gnotobiotic piglets from NoV-induced gastroenteritis. Such a study will facilitate the future clinical trial of NoV-specific IgY in humans.

Chicken as a "factory" for large-scale production of antigen-specific IgY. Antibody-based passive immunization and therapy has been shown to be an effective strategy to prevent infectious diseases in many animals (113). However, preparation of serum antibody from mammals is expensive and time consuming. Thus, large-scale application of serum antibody has been limited. IgY egg yolk immunoglobulins derived from hyperimmunized hens represent an economically feasible and practical strategy which has been explored for the passive treatment of enteric diseases. Chicken IgY production is a much easier, faster, and cheaper method for polyclonal antibody production than from any other sources. It is easy to raise chickens and collect eggs without involvement of any stressful procedures (such as bleeding). White leghorn chickens are highly productive in laying eggs, and they can continuously produce eggs containing antigen-specific antibodies in their yolks for a long time period after immunization (113). Nguyen et al. (2010) demonstrated that chicken usually lays 280eggs/year and each egg yolk normally contains 150–200 mg

of IgY, which has 2–10% antigen-specific antibodies (81). Furthermore, extraction of antibody from egg yolk is simple and non-invasive without affecting the immunized chickens. Therefore, a chicken is referred as a small “factory” for antibody production.

In order to be used as immunological supplements in infant formulas and other foods, it is important to investigate the stability of IgY during storage or following processing methods, involving thermal treatments, such as pasteurization, sterilization, or spray drying. Based on the HBGA blocking assay, human NoV-specific IgY remains stable at temperature below 70 °C. However, the receptor blocking activity of IgY significantly decreased when temperature reached above 75 °C, suggesting that IgY may be denatured at this temperature. This, it should be safe for pasteurization (below 72^{o}C) of IgY for human consumption.

And it is also the important consideration when we want to preserve the immunoprophylactic or therapeutic potential of IgY for human clinical trials.For oral administration, IgY should ideally be stable in acid or alkaline environment since it must go through human digestive system. Our results showed that the receptor blocking activity of IgY decreased at pH below 3 or above 10. Since the stomach pH is around 2-3, it may be necessary to encapsulate the IgY in acid resistant capsules so that it can be released in intestines for virus neutralization. For example, Chang et al. (1999) demonstrated that addition of sugars, glycerol, or glycine to immunoglobulin solutions was effective to protect IgYs. In addition, film coating with gum arabic was proven to be effective in reducing the degree of hydrolysis (23).

In summary, we developed a highly efficient bioreactor to generate a high titer of human NoV-sepcific IgY by vaccination of hens with rVSV-VP1. The human

NoV-sepcific IgY was biologically active in capturing human NoV antigen and blocking the interaction between VLPs and HBGA receptors. This study will facilitate the large scale production and purification of human NoV-specific IgY for virus detection, disease diagnosis, passive immunization, and therapy.

CHAPTER 3

CONCLUSION

First, this study demonstrated that rVSV-VP1 replicated efficiently in chicken cell lines in vitro and VP1 protein can be highly expressed by VSV vector. A high level of human NoV-specific IgY can be produced in egg yolks by using the live attenuated rVSV-VP1 as an antigen.

Second, this study demonstrated that intramuscular vaccination of hens with rVSV-VP1 triggered significantly higher human NoV-specific IgY than the combination of intramuscular and nasal drop vaccination route.

Third, this study found that the amount of human NoV-specific IgY generated by intramuscular vaccination could reach 4.8 mg per yolk at week 4 post-vaccination. The percentage of human NoV-specific IgY in total IgY was approximately 5%. Fourth, this study demonstrated that the purified human NoV-specific IgY was biologically active. The purified IgY strongly reacted with human NoV virus-like particles (VLPs) by both ELISA and Western blot. Most importantly, human NoV-specific IgY was highly capable of blocking the binding of human NoV VLPs to the histo-blood group antigen (HBGA) receptors.

Fifth, this study investigated the thermal and pH stability of human NoV-specific IgY using HBGA blocking assay. The human NoV-specific IgY remained stable at temperature below 70 ^{o}C and pH from 4 to 9.

Collectively, these results demonstrate that (i) recombinant rVSV-VP1 is a safe and highly efficient antigen to produce human NoV-specific IgYin chickens; (ii) human NoV-specific IgY is biologically active and highly capable of blocking the interaction between human NoV VLPs and HBGA receptors; and (iii) chicken IgY could be a practical strategy for large-scale production of anti-human NoV antibodies for potential use in passive immunization against human NoV infection as well as for therapeutic and diagnostic purposes.

LIST OF REFERENCES

1. **Abraham G, Banerjee AK.** 1976. Sequential transcription of the genes of vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America **73:**1504-1508.
2. **Adler JL, Zickl R.** 1969. Winter vomiting disease. The Journal of infectious diseases **119:**668-673.
3. **Amaral JA, Tino De Franco M, Carneiro-Sampaio MM, Carbonare SB.** 2002. Anti-enteropathogenic Escherichia coli immunoglobulin Y isolated from eggs laid by immunised Leghorn chickens. Research in veterinary science **72:**229-234.
4. **Atmar RL, Estes MK.** 2001. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clinical microbiology reviews **14:**15-37.
5. **Ball JM, Graham DY, Opekun AR, Gilger MA, Guerrero RA, Estes MK.** 1999. Recombinant Norwalk virus-like particles given orally to volunteers: phase I study. Gastroenterology **117:**40-48.
6. **Ball JM, Hardy ME, Atmar RL, Conner ME, Estes MK.** 1998. Oral immunization with recombinant Norwalk virus-like particles induces a systemic and mucosal immune response in mice. Journal of virology **72:**1345-1353.
7. **Ball LA.** 1977. Transcriptional mapping of vesicular stomatitis virus in vivo. Journal of virology **21:**411-414.
8. **Ball LA, White CN.** 1976. Order of transcription of genes of vesicular stomatitis virus. Proceedings of the National Academy of Sciences of the United States of America **73:**442-446.
9. **Baxter D.** 2007. Active and passive immunity, vaccine types, excipients and licensing. Occupational medicine **57:**552-556.
10. **Becker KM, Moe CL, Southwick KL, MacCormack JN.** 2000. Transmission of Norwalk virus during football game. The New England journal of medicine **343:**1223-1227.
11. **Bertolotti-Ciarlet A, Crawford SE, Hutson AM, Estes MK.** 2003. The 3' end of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein. Journal of virology **77:**11603-11615.

12. **Bidawid S, Malik N, Adegbunrin O, Sattar SA, Farber JM.** 2004. Norovirus cross-contamination during food handling and interruption of virus transfer by hand antisepsis: experiments with feline calicivirus as a surrogate. Journal of food protection **67:**103-109.
13. **Brugha R, Vipond IB, Evans MR, Sandifer QD, Roberts RJ, Salmon RL, Caul EO, Mukerjee AK.** 1999. A community outbreak of food-borne small round-structured virus gastroenteritis caused by a contaminated water supply. Epidemiology and infection **122:**145-154.
14. **Burley RW, Cook WH.** 1961. Isolation and composition of avian egg yolk granules and their constituent alpha- and beta-lipovitellins. Canadian journal of biochemistry and physiology **39:**1295-1307.
15. **Camenisch G, Tini M, Chilov D, Kvietikova I, Srinivas V, Caro J, Spielmann P, Wenger RH, Gassmann M.** 1999. General applicability of chicken egg yolk antibodies: the performance of IgY immunoglobulins raised against the hypoxia-inducible factor 1alpha. FASEB journal : official publication of the Federation of American Societies for Experimental Biology **13:**81-88.
16. **Carlander D, Kollberg H, Wejaker PE, Larsson A.** 2000. Peroral immunotherapy with yolk antibodies for the prevention and treatment of enteric infections. Immunologic research **21:**1-6.
17. **Carter MJ.** 2005. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. Journal of applied microbiology **98:**1354-1380.
18. **Casadevall A, Dadachova E, Pirofski LA.** 2004. Passive antibody therapy for infectious diseases. Nature reviews. Microbiology **2:**695-703.
19. **CDC.** Centers for disease control and prevention 2010. CDC Reports 1 in 6 Get Sick from Foodborne Illnesses Each Year. http://www.cdc.gov/media/pressrel/2010/r101215.html.
20. **CDC.** Centers for disease control and prevention 2011. Updated norovirus outbreak management and disease prevention guidelines. MMWR**:**60:61-15.
21. **Chalghoumi R, Marcq C, Thewis A, Portetelle D, Beckers Y.** 2009. Effects of feed supplementation with specific hen egg yolk antibody (immunoglobin Y) on Salmonella species cecal colonization and growth performances of challenged broiler chickens. Poultry science **88:**2081-2092.
22. **Chan MC, Wong YP, Leung WK.** 2007. Cell culture assay for human noroviruses. Emerging infectious diseases **13:**1117; author reply 1117-1118.
23. **Chang HM, Ou-Yang RF, Chen YT, Chen CC.** 1999. Productivity and some properties of immunoglobulin specific against Streptococcus mutans serotype c in chicken egg yolk (IgY). Journal of agricultural and food chemistry **47:**61-66.
24. **Cheek JE, Young P, Branch L, Dupnik KM, Kelly ST, Sharp JM, Toney DM, Bresee JS, Monroe SS, Beard RS, Bulens S, Leman R, Centers for Disease C, Prevention.** 2002. Norwalk-like virus-associated gastroenteritis in a large, high-density encampment--Virginia, July 2001. MMWR. Morbidity and mortality weekly report **51:**661-663.
25. **Chucri TM, Monteiro JM, Lima AR, Salvadori ML, Kfoury JR, Jr., Miglino MA.** 2010. A review of immune transfer by the placenta. Journal of reproductive immunology **87:**14-20.

26. **Cliver DO.** 1997. Virus transmission via food. World health statistics quarterly. Rapport trimestriel de statistiques sanitaires mondiales **50:**90-101.
27. **Cooper HM, Paterson Y.** 2009. Production of polyclonal antisera. Current protocols in neuroscience / editorial board, Jacqueline N. Crawley ... [et al.] **Chapter 5:**Unit 5 5.
28. **Dai YC, Wang YY, Zhang XF, Tan M, Xia M, Wu XB, Jiang X, Nie J.** 2012. Evaluation of anti-norovirus IgY from egg yolk of chickens immunized with norovirus P particles. Journal of virological methods **186:**126-131.
29. **Davalos A, Shuaib A, Wahlgren NG.** 2000. Neurotransmitters and pathophysiology of stroke: evidence for the release of glutamate and other transmitters/mediators in animals and humans. Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association **9:**2-8.
30. **DiLorenzo N, Dahlen CR, Diez-Gonzalez F, Lamb GC, Larson JE, DiCostanzo A.** 2008. Effects of feeding polyclonal antibody preparations on rumen fermentation patterns, performance, and carcass characteristics of feedlot steers. Journal of animal science **86:**3023-3032.
31. **Doultree JC, Druce JD, Birch CJ, Bowden DS, Marshall JA.** 1999. Inactivation of feline calicivirus, a Norwalk virus surrogate. The Journal of hospital infection **41:**51-57.
32. **Duizer E, Bijkerk P, Rockx B, De Groot A, Twisk F, Koopmans M.** 2004. Inactivation of caliciviruses. Applied and environmental microbiology **70:**4538-4543.
33. **Ebina T.** 1996. Prophylaxis of rotavirus gastroenteritis using immunoglobulin. Archives of virology. Supplementum **12:**217-223.
34. **Erhard MH, Ozpinar H, Bilal T, Abas I, Kutay C, Eseceli H, Stangassinger M.** 2000. The humoral immune response and the productivity of laying hens kept on the ground or in cages. Alternatives to laboratory animals : ATLA **28:**699-705.
35. **Estes MK, Prasad BV, Atmar RL.** 2006. Noroviruses everywhere: has something changed? Current opinion in infectious diseases **19:**467-474.
36. **Feng K, Divers E, Ma Y, Li J.** 2011. Inactivation of a human norovirus surrogate, human norovirus virus-like particles, and vesicular stomatitis virus by gamma irradiation. Applied and environmental microbiology **77:**3507-3517.
37. **Fryer J, Firca J, Leventhal J, Blondin B, Malcolm A, Ivancic D, Gandhi R, Shah A, Pao W, Abecassis M, Kaufman D, Stuart F, Anderson B.** 1999. IgY antiporcine endothelial cell antibodies effectively block human antiporcine xenoantibody binding. Xenotransplantation **6:**98-109.
38. **Gassmann M, Thommes P, Weiser T, Hubscher U.** 1990. Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. FASEB journal : official publication of the Federation of American Societies for Experimental Biology **4:**2528-2532.
39. **Gaudier M, Gaudin Y, Knossow M.** 2002. Crystal structure of vesicular stomatitis virus matrix protein. The EMBO journal **21:**2886-2892.
40. **Glass RI, Parashar UD, Estes MK.** 2009. Norovirus gastroenteritis. The New England journal of medicine **361:**1776-1785.

41. **Goodgame R.** 2006. Norovirus gastroenteritis. Current gastroenterology reports **8:**401-408.
42. **Gospodarek E, Zalas-Wiecek P.** 2009. [Noroviruses--tactic of spread]. Przeglad epidemiologiczny **63:**5-9.
43. **Graham DY, Jiang X, Tanaka T, Opekun AR, Madore HP, Estes MK.** 1994. Norwalk virus infection of volunteers: new insights based on improved assays. The Journal of infectious diseases **170:**34-43.
44. **Hardy ME.** 2005. Norovirus protein structure and function. FEMS microbiology letters **253:**1-8.
45. **Harris JP, Edmunds WJ, Pebody R, Brown DW, Lopman BA.** 2008. Deaths from norovirus among the elderly, England and Wales. Emerging infectious diseases **14:**1546-1552.
46. **Hatta H, Tsuda K, Akachi S, Kim M, Yamamoto T.** 1993. Productivity and some properties of egg yolk antibody (IgY) against human rotavirus compared with rabbit IgG. Bioscience, biotechnology, and biochemistry **57:**450-454.
47. **Hatta H, Tsuda K, Akachi S, Kim M, Yamamoto T, Ebina T.** 1993. Oral passive immunization effect of anti-human rotavirus IgY and its behavior against proteolytic enzymes. Bioscience, biotechnology, and biochemistry **57:**1077-1081.
48. **Hatta H, Tsuda K, Ozeki M, Kim M, Yamamoto T, Otake S, Hirasawa M, Katz J, Childers NK, Michalek SM.** 1997. Passive immunization against dental plaque formation in humans: effect of a mouth rinse containing egg yolk antibodies (IgY) specific to Streptococcus mutans. Caries research **31:**268-274.
49. **Hedberg CW, Osterholm MT.** 1993. Outbreaks of food-borne and waterborne viral gastroenteritis. Clinical microbiology reviews **6:**199-210.
50. **Holt D, Brown J, Terrill K, Goldsby R, Meyers RL, Heximer J, Nordfors B, Slayton WB.** 2003. Response to intravenous immunoglobulin predicts splenectomy response in children with immune thrombocytopenic purpura. Pediatrics **111:**87-90.
51. **Huang JC, Wun WS, Goldsby JS, Wun IC, Falconi SM, Wu KK.** 2003. Prostacyclin enhances embryo hatching but not sperm motility. Human reproduction **18:**2582-2589.
52. **Hutson AM, Atmar RL, Estes MK.** 2004. Norovirus disease: changing epidemiology and host susceptibility factors. Trends in microbiology **12:**279-287.
53. **Hutson AM, Atmar RL, Graham DY, Estes MK.** 2002. Norwalk virus infection and disease is associated with ABO histo-blood group type. The Journal of infectious diseases **185:**1335-1337.
54. **Hutson AM, Atmar RL, Marcus DM, Estes MK.** 2003. Norwalk virus-like particle hemagglutination by binding to h histo-blood group antigens. Journal of virology **77:**405-415.
55. **Ikemori Y, Kuroki M, Peralta RC, Yokoyama H, Kodama Y.** 1992. Protection of neonatal calves against fatal enteric colibacillosis by administration of egg yolk powder from hens immunized with K99-piliated enterotoxigenic Escherichia coli. American journal of veterinary research **53:**2005-2008.

56. **Jiang X, Wang M, Wang K, Estes MK.** 1993. Sequence and genomic organization of Norwalk virus. Virology **195:**51-61.
57. **JL F.** 2011. Foodborne illness estimates revised; major, minor safety changes pending. Microbe **6 (2):**54-55.
58. **Kalhoro NH, Veits J, Rautenschlein S, Zimmer G.** 2009. A recombinant vesicular stomatitis virus replicon vaccine protects chickens from highly pathogenic avian influenza virus (H7N1). Vaccine **27:**1174-1183.
59. **Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM.** 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. Journal of virology **10:**1075-1081.
60. **Kaplan JE, Feldman R, Campbell DS, Lookabaugh C, Gary GW.** 1982. The frequency of a Norwalk-like pattern of illness in outbreaks of acute gastroenteritis. American journal of public health **72:**1329-1332.
61. **Keller MA, Stiehm ER.** 2000. Passive immunity in prevention and treatment of infectious diseases. Clinical microbiology reviews **13:**602-614.
62. **Koopmans M, Duizer E.** 2004. Foodborne viruses: an emerging problem. International journal of food microbiology **90:**23-41.
63. **Koopmans M, von Bonsdorff CH, Vinje J, de Medici D, Monroe S.** 2002. Foodborne viruses. FEMS microbiology reviews **26:**187-205.
64. **Kovacs-Nolan J, Mine Y.** 2005. Microencapsulation for the gastric passage and controlled intestinal release of immunoglobulin Y. Journal of immunological methods **296:**199-209.
65. **Kovacs-Nolan J, Mine Y.** 2012. Egg yolk antibodies for passive immunity. Annual review of food science and technology **3:**163-182.
66. **Kuroki M, Ikemori Y, Yokoyama H, Peralta RC, Icatlo FC, Jr., Kodama Y.** 1993. Passive protection against bovine rotavirus-induced diarrhea in murine model by specific immunoglobulins from chicken egg yolk. Veterinary microbiology **37:**135-146.
67. **Kuroki M, Ohta M, Ikemori Y, Peralta RC, Yokoyama H, Kodama Y.** 1994. Passive protection against bovine rotavirus in calves by specific immunoglobulins from chicken egg yolk. Archives of virology **138:**143-148.
68. **Kweon CH, Kwon BJ, Woo SR, Kim JM, Woo GH, Son DH, Hur W, Lee YS.** 2000. Immunoprophylactic effect of chicken egg yolk immunoglobulin (Ig Y) against porcine epidemic diarrhea virus (PEDV) in piglets. The Journal of veterinary medical science / the Japanese Society of Veterinary Science **62:**961-964.
69. **Larsson A, Balow RM, Lindahl TL, Forsberg PO.** 1993. Chicken antibodies: taking advantage of evolution--a review. Poultry science **72:**1807-1812.
70. **Larsson A, Karlsson-Parra A, Sjoquist J.** 1991. Use of chicken antibodies in enzyme immunoassays to avoid interference by rheumatoid factors. Clinical chemistry **37:**411-414.
71. **Lay MK, Atmar RL, Guix S, Bharadwaj U, He H, Neill FH, Sastry KJ, Yao Q, Estes MK.** 2010. Norwalk virus does not replicate in human macrophages or dendritic cells derived from the peripheral blood of susceptible humans. Virology **406:**1-11.

72. **Letchworth GJ, Rodriguez LL, Del cbarrera J.** 1999. Vesicular stomatitis. Veterinary journal **157:**239-260.
73. **Leung WK, Chan PK, Lee NL, Sung JJ.** 2010. Development of an in vitro cell culture model for human noroviruses and its clinical application. Hong Kong medical journal = Xianggang yi xue za zhi / Hong Kong Academy of Medicine **16:**18-21.
74. **Li J, Predmore A, Divers E, Lou F.** 2012. New interventions against human norovirus: progress, opportunities, and challenges. Annual review of food science and technology **3:**331-352.
75. **Lilius EM, Marnila P.** 2001. The role of colostral antibodies in prevention of microbial infections. Current opinion in infectious diseases **14:**295-300.
76. **Ma Y, Li J.** 2011. Vesicular stomatitis virus as a vector to deliver virus-like particles of human norovirus: a new vaccine candidate against an important noncultivable virus. Journal of virology **85:**2942-2952.
77. **Marks PJ, Vipond IB, Regan FM, Wedgwood K, Fey RE, Caul EO.** 2003. A school outbreak of Norwalk-like virus: evidence for airborne transmission. Epidemiology and infection **131:**727-736.
78. **Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV.** 1999. Food-related illness and death in the United States. Emerging infectious diseases **5:**607-625.
79. **Mohammed SM, Morrison S, Wims L, Trinh KR, Wildeman AG, Bonselaar J, Etches RJ.** 1998. Deposition of genetically engineered human antibodies into the egg yolk of hens. Immunotechnology : an international journal of immunological engineering **4:**115-125.
80. **Morrison SL, Mohammed MS, Wims LA, Trinh R, Etches R.** 2002. Sequences in antibody molecules important for receptor-mediated transport into the chicken egg yolk. Molecular immunology **38:**619-625.
81. **Nguyen HH, Tumpey TM, Park HJ, Byun YH, Tran LD, Nguyen VD, Kilgore PE, Czerkinsky C, Katz JM, Seong BL, Song JM, Kim YB, Do HT, Nguyen T, Nguyen CV.** 2010. Prophylactic and therapeutic efficacy of avian antibodies against influenza virus H5N1 and H1N1 in mice. PloS one **5:**e10152.
82. **Parrino TA, Schreiber DS, Trier JS, Kapikian AZ, Blacklow NR.** 1977. Clinical immunity in acute gastroenteritis caused by Norwalk agent. The New England journal of medicine **297:**86-89.
83. **Patterson R, Youngner JS, Weigle WO, Dixon FJ.** 1962. Antibody production and transfer to egg yolk in chickens. Journal of immunology **89:**272-278.
84. **Peralta RC, Yokoyama H, Ikemori Y, Kuroki M, Kodama Y.** 1994. Passive immunisation against experimental salmonellosis in mice by orally administered hen egg-yolk antibodies specific for 14-kDa fimbriae of Salmonella enteritidis. Journal of medical microbiology **41:**29-35.
85. **Polson A, Coetzer T, Kruger J, von Maltzahn E, van der Merwe KJ.** 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. Immunological investigations **14:**323-327.
86. **Raja Chalghoumi YB, Daniel Portelle, Andre Thewis.** 2009. Hen egg yolk antibodies (IgY), production and use for passive immunization against

bacterial enteric infections in chicken: a review. Biotechnol. Agron. Soc. Environ **13 （2）:**295-308.

87. **Rockx B, De Wit M, Vennema H, Vinje J, De Bruin E, Van Duynhoven Y, Koopmans M.** 2002. Natural history of human calicivirus infection: a prospective cohort study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **35:**246-253.
88. **Rose ME, Orlans E.** 1981. Immunoglobulins in the egg, embryo and young chick. Developmental and comparative immunology **5:**15-20.
89. **Rose ME, Orlans E, Buttress N.** 1974. Immunoglobulin classes in the hen's egg: their segregation in yolk and white. European journal of immunology **4:**521-523.
90. **Rzezutka A, Cook N.** 2004. Survival of human enteric viruses in the environment and food. FEMS microbiology reviews **28:**441-453.
91. **Schade R, Calzado EG, Sarmiento R, Chacana PA, Porankiewicz-Asplund J, Terzolo HR.** 2005. Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and human and veterinary medicine. Alternatives to laboratory animals : ATLA **33:**129-154.
92. **Schade R, Hlinak A.** 1996. Egg Yolk Antibodies, State of the Art and Future Prospects. Altex **13:**5-9.
93. **Schaden W, Thiele R, Kolpl C, Pusch M, Nissan A, Attinger CE, Maniscalco-Theberge ME, Peoples GE, Elster EA, Stojadinovic A.** 2007. Shock wave therapy for acute and chronic soft tissue wounds: a feasibility study. The Journal of surgical research **143:**1-12.
94. **Sharma JM.** 1999. Introduction to poultry vaccines and immunity. Advances in veterinary medicine **41:**481-494.
95. **Shimizu A, Tepler I, Benfey PN, Berenstein EH, Siraganian RP, Leder P.** 1988. Human and rat mast cell high-affinity immunoglobulin E receptors: characterization of putative alpha-chain gene products. Proceedings of the National Academy of Sciences of the United States of America **85:**1907-1911.
96. **Shimizu M, Nagashima H, Hashimoto K.** 1993. Comparative studies in molecular stability of immunoglobulin G from different species. Comparative biochemistry and physiology. B, Comparative biochemistry **106:**255-261.
97. **Sun S, Mo W, Ji Y, Liu S.** 2001. Preparation and mass spectrometric study of egg yolk antibody (IgY) against rabies virus. Rapid communications in mass spectrometry : RCM **15:**708-712.
98. **Tan M, Jiang X.** 2005. Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle. Trends in microbiology **13:**285-293.
99. **Tesar DB, Cheung EJ, Bjorkman PJ.** 2008. The chicken yolk sac IgY receptor, a mammalian mannose receptor family member, transcytoses IgY across polarized epithelial cells. Molecular biology of the cell **19:**1587-1593.
100. **Teunis PF, Moe CL, Liu P, Miller SE, Lindesmith L, Baric RS, Le Pendu J, Calderon RL.** 2008. Norwalk virus: how infectious is it? Journal of medical virology **80:**1468-1476.
101. **Thornton AC, Jennings-Conklin KS, McCormick MI.** 2004. Noroviruses: agents in outbreaks of acute gastroenteritis. Disaster management & response : DMR : an official publication of the Emergency Nurses Association **2:**4-9.

102. **Tini M, Jewell UR, Camenisch G, Chilov D, Gassmann M.** 2002. Generation and application of chicken egg-yolk antibodies. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology **131:**569-574.
103. **Treanor JJ DR.** 2000. Norwalk Virus and Other Caliciviruses. In: Mandell GL, Bennett JE, 5th Edition ed. Principles and Practice of Infectious Diseases **Vol 2:**1949-1956.
104. **Tressler RL, Roth TF.** 1987. IgG receptors on the embryonic chick yolk sac. The Journal of biological chemistry **262:**15406-15412.
105. **Tsubokura K, Berndtson E, Bogstedt A, Kaijser B, Kim M, Ozeki M, Hammarstrom L.** 1997. Oral administration of antibodies as prophylaxis and therapy in Campylobacter jejuni-infected chickens. Clinical and experimental immunology **108:**451-455.
106. **Turcios RM, Widdowson MA, Sulka AC, Mead PS, Glass RI.** 2006. Reevaluation of epidemiological criteria for identifying outbreaks of acute gastroenteritis due to norovirus: United States, 1998-2000. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **42:**964-969.
107. **Turcios-Ruiz RM, Axelrod P, St John K, Bullitt E, Donahue J, Robinson N, Friss HE.** 2008. Outbreak of necrotizing enterocolitis caused by norovirus in a neonatal intensive care unit. The Journal of pediatrics **153:**339-344.
108. **Vega C, Bok M, Chacana P, Saif L, Fernandez F, Parreno V.** 2011. Egg yolk IgY: protection against rotavirus induced diarrhea and modulatory effect on the systemic and mucosal antibody responses in newborn calves. Veterinary immunology and immunopathology **142:**156-169.
109. **Wang Q, Hou XJ, Cai K, Li T, Liu YN, Tu W, Xiao L, Bao SZ, Shi J, Gao X, Liu H, Tian RM, Wang H.** 2010. Passive protection of purified yolk immunoglobulin administered against Shiga toxin 1 in mouse models. Canadian journal of microbiology **56:**1003-1010.
110. **Warr GW, Magor KE, Higgins DA.** 1995. IgY: clues to the origins of modern antibodies. Immunology today **16:**392-398.
111. **Widdowson MA, Sulka A, Bulens SN, Beard RS, Chaves SS, Hammond R, Salehi ED, Swanson E, Totaro J, Woron R, Mead PS, Bresee JS, Monroe SS, Glass RI.** 2005. Norovirus and foodborne disease, United States, 1991-2000. Emerging infectious diseases **11:**95-102.
112. **Wiedemann V, Linckh E, Kuhlmann R, Schmidt P, Losch U.** 1991. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. V. In vivo studies on protective effects against Escherichia coli diarrhea in pigs. Zentralblatt fur Veterinarmedizin. Reihe B. Journal of veterinary medicine. Series B **38:**283-291.
113. **Xu Y, Li X, Jin L, Zhen Y, Lu Y, Li S, You J, Wang L.** 2011. Application of chicken egg yolk immunoglobulins in the control of terrestrial and aquatic animal diseases: a review. Biotechnology advances **29:**860-868.
114. **Yokoyama H, Peralta RC, Diaz R, Sendo S, Ikemori Y, Kodama Y.** 1992. Passive protective effect of chicken egg yolk immunoglobulins against experimental enterotoxigenic Escherichia coli infection in neonatal piglets. Infection and immunity **60:**998-1007.

115. **Yokoyama H, Peralta RC, Sendo S, Ikemori Y, Kodama Y.** 1993. Detection of passage and absorption of chicken egg yolk immunoglobulins in the gastrointestinal tract of pigs by use of enzyme-linked immunosorbent assay and fluorescent antibody testing. American journal of veterinary research **54:**867-872.
116. **Yokoyama H, Umeda K, Peralta RC, Hashi T, Icatlo FC, Jr., Kuroki M, Ikemori Y, Kodama Y.** 1998. Oral passive immunization against experimental salmonellosis in mice using chicken egg yolk antibodies specific for Salmonella enteritidis and S. typhimurium. Vaccine **16:**388-393.
117. **Yolken RH, Leister F, Wee SB, Miskuff R, Vonderfecht S.** 1988. Antibodies to rotaviruses in chickens' eggs: a potential source of antiviral immunoglobulins suitable for human consumption. Pediatrics **81:**291-295.
118. **Zhen YH, Jin LJ, Guo J, Li XY, Lu YN, Chen J, Xu YP.** 2008. Characterization of specific egg yolk immunoglobulin (IgY) against mastitis-causing Escherichia coli. Veterinary microbiology **130:**126-133.
119. **Zheng DP, Ando T, Fankhauser RL, Beard RS, Glass RI, Monroe SS.** 2006. Norovirus classification and proposed strain nomenclature. Virology **346:**312-323.