12-2014

Acanthamoeba spp. as Reservoirs for Transmission of Norovirus

Tun-Yun Hsueh
University of Arkansas, Fayetteville

Acanthamoeba spp. as Reservoirs for Transmission of Norovirus

Acanthamoeba spp. as Reservoirs for Transmission of Norovirus

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Food Science

By

Tun-Yun Hsueh
National Taiwan University
Bachelor of Science in Medical Technology, 2000
National Taiwan University
Master of Science in Medical Technology, 2004

December 2014
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

_____

Dr. Kristen E. Gibson
Thesis Director

_____

Dr. Steven C. Ricke
Committee Member

_____

Dr. Jeffrey Silberman
Committee Member

Abstract

Human noroviruses (HuNoV) are the most common cause of foodborne disease outbreaks in the United States and the most common food commodities implicated in HuNoV outbreaks are leafy greens; however, the vehicle of transmission and point of contamination are often unknown. Here, we hypothesize that common free-living amoebae (FLA) ubiquitous in the environment may act as reservoirs of HuNoV and facilitate the transmission of these pathogens to fresh produce. The objective of this research was to first evaluate the interaction/association between HuNoV surrogates and *Acanthamoeba* by incubating them together and analyzing virus titer associated with amoeba through an 8 day period and a complete life cycle. Also, the location of associated virus in/on amoeba was investigated. Last, the transfer of virus-associated amoeba from the environment to fresh produce was evaluated.

In conclusion, *A. castellanii* can interact with a HuNoV surrogate, murine norovirus type 1 (MNV-1) and associate with MNV-1 for 8 days and even through the complete amoeba life cycle. In addition while viruses were still infectious, immunofluorescence staining results indicated that MNV-1 changed its location from amoeba surface to inside amoeba over 24 hours. Finally, the transfer of virus-associated amoeba from water to fresh produce was notable. Based on the data we presented, *A. castellanii* appears to have potential for carrying MNV-1 and transfer it to fresh

produce in water and therefore, possibly playing a role in the transmission of HuNoV from aquatic environment to fresh produce.

The findings of this study are significant in understanding the HuNoV survival in the environment and for the future control of HuNoV outbreaks where FLA may serve as important reservoirs.

Acknowledgments

I would like to thank my committee members Dr. Gibson, Dr. Ricke and Dr. Silberman for their continuous support and guidance throughout the development of my thesis research.

Dr. Gibson, you gave me the chance to be one of the graduate students in Food Science Department by accepting me as your first student at the University of Arkansas. When my research was confronted with obstacles, you were always there to provide guidance and keep me in the best direction for my project. When I was struggling through my thesis writing, you provided me with detailed suggestions and patiently worked with me as my writing progressed. You always believed in me and encouraged me even when I had doubts about myself. I feel blessed to be one of your students and I sincerely appreciate your unwavering patience and encouragement.

Dr. Ricke, you introduced me to Dr. Gibson when I applied for the graduate program in Food Science. This turned out to be a wonderful relationship between Dr. Gibson and I, and an enriching experience for my graduate student life. Thank you for your brilliant thoughts during the discussion of my thesis project and journal club. Your suggestions were precious and did help me make this research complete.

Dr. Silberman, you introduced me to the world of amoeba and demonstrated and trained me on the isolation and culture of *Acanthamoeba*. Thank you for your knowledge in protozoa as well as your support and guidance in lab skills which was also a crucial key to this thesis development.

I would also like to thank you, Giselle Almeida, for helping me when I was new to the lab as well as keeping the lab up and running. You are like a big sister in the lab and always willing to provide support and take care of me. Thank you to Dr. Corliss O'Bryan for helping me access experimental materials needed during research.

Additional thanks to Sabastine E. Arthur for covering and helping my experiments during the time when I could not make it. Thank you to all the members in food safety lab who were always willing to help in my learning process and to the wonderful faculty and staff of the Food Science department for their support and involvement with their students.

Dedication

This thesis is dedicated to my husband, Chung-Lin Huang for making me free of worries about life during my graduate student life and keeping me company in all the hard work and late nights during my research.

Table of Contents

Chapter 1: Overall Introduction

I. Foodborne Pathogens

Foodborne pathogens are microorganisms which cause diseases in humans through ingestion of contaminated foods. They include bacteria, viruses, protozoa and other microorganisms. Foodborne pathogens are a leading cause of illness in the United States. In 2011, the Centers for Disease Control and Prevention (CDC) estimated that there were around 48 million cases per year of illnesses due to foodborne agents, with 128,000 hospitalizations, and 6,000 deaths in the United States each year (CDC, 2011). *Campylobacter jejuni*, *Salmonella*, and *Escherichia coli* O157:H7 are the most common bacterial pathogens, and human noroviruses are the most common viral pathogens that cause foodborne illnesses.

II. Human Norovirus

Introduction

Noroviruses (NoVs) belong to a highly genetically and antigenically diverse genus, *Norovirus,* of the *Caliciviridae* family. Norovirus genomes are composed of a single, linear, and positive-sense RNA of 7.4-7.7 kilobases which is usually organized into three open reading frames (ORF 1-3). These three ORFs encode 7 nonstructural proteins (NPs), the major structural protein VP1, and the minor structural protein VP2, respectively. VP1 and VP2 comprise the non-enveloped, icosahedral protein capsid, which encloses the viral RNA genome. Virions are

nonenveloped (not surrounded by a lipid bilayer membrane) and are approximately 27 to 35nm in size (Green, 2007).

Noroviruses are divided into different genogroups according to the genetic similarity of highly conserved regions of their genomes such as the genes encoding RNA-dependent RNA polymerase and the VP1 protein (Green, 2007). In addition, different virus strains in the same genogroup share at least 60% amino acid sequence identity of the major capsid protein VP1 (Hutson et al., 2004). Five genogroups (GI, GII, GIII, GIV, and GV) have been identified; GI, GII and GIV are associated with human diseases while GII and GIV also contain some porcine-specific genotypes and a feline-specific genotype, respectively. Finally, GIII and GV are associated with bovine and murine species, respectively (Glass et al., 2009).

Noroviruses associated with human disease are called human noroviruses (HuNoV). As described above, HuNoVs include three genogroups, GI, GII and GIV, which are further divided into more than 26 different genotypes (Bull et al., 2010; Morillo et al., 2011). In the last 15 years, GII has been responsible for the majority of HuNoV outbreaks with GII.4 being the most common strain accounting for 62% of all norovirus outbreak globally (Bull et al., 2010; Bull and White, 2011).

Transmission and pathogenesis

With a non-enveloped capsid, HuNoVs are very stable on inanimate surfaces and resistant to conventional cleaning agents (e.g., chlorine bleach, quaternary ammonium compounds) (Keswick et al., 1985; Duizer et al., 2004). Moreover, only a low virus dose (18-1,000 virus particles) is needed to cause infection (Teunis et al., 2008). According to the National Institute of Allergy and Infectious Diseases classification of pathogens important for

biodefense (NIAID Biodefense Research), HuNoVs are classified as Category B potential bioterrorism agents since they have ideal properties as pathogens of enteric outbreaks. Transmission occurs primarily through exposure to contaminated food or water, person-to-person contact, aerosolized particles from vomitus, and the contact with fomites (i.e. door handles, toilets, tables, and elevator buttons.) (Lopman et al., 2012). Symptoms of HuNoV infections include nausea, vomiting, abdominal cramps, and non-bloody diarrhea. Though HuNoV infections are typically acute and self-limiting, the infections can be much more serious in infants, young children, elderly and immunocompromised patients resulting in prolonged infection that may require hospitalization and possibly result in death related to dehydration (Patel et al., 2009). Moreover, because of the recent improvements in detection methods and technology, HuNoVs have been reportedly associated with several clinical symptoms other than gastroenteritis including encephalopathy (Karst, 2010; Trivedi et al., 2012).

Foods implicated in outbreaks caused by human noroviruses

Food service workers who carry HuNoVs at restaurant, banquet hall and catered events are common causes of foodborne norovirus outbreak (Fankhauser et al., 1998). In addition, possible food vehicles of HuNoV can also get contaminated during growing, harvest, transport and processing (Ethelberg et al., 2010). Ready to eat (RTE) food and food with minimal processing are most commonly associated with HuNoV outbreak, and those foods include bivalve molluscan shellfish, fresh produce, deli sandwiches, and baked goods. Moreover, the most frequent food commodity implicated in HuNoV outbreaks is contaminated leafy greens which are responsible for an estimated 33% of the reported norovirus outbreaks followed by fruits/nuts (16%), and mollusks (13%) (Hall et al., 2012).

Socio-economic impact of human noroviruses

In the past, bacterial infections were thought to be more prevalent because few public health and clinical laboratories had the capability to test for HuNoVs, and no active surveillance program was in place for this particular etiologic agent. Over the past decade, because of improved detection technologies, HuNoV infections have been determined to be the most prevalent cause of foodborne illnesses (Koo et al., 2010). Also, due to the high rate of transmission, the CDC considers HuNoVs to be the most common cause of foodborne disease outbreaks in the United States (CDC, 2009). It is estimated that HuNoVs cause over one million hospitalizations and 200,000 deaths of young children in developing countries annually (Patel et al., 2008). In addition, Scallan et al. (2011) estimated that there are 5.5 million foodborne norovirus illnesses in the United States each year resulting in 15,000 hospitalization and 150 deaths and up to 2 million US dollar in related health care costs. For instance, the socio-economic burden of a single nosocomial HuNoV outbreak in a healthcare setting costs nearly $660,000 in lost revenue, sick leave, and cleaning expenses (Johnston et al., 2007).

III. Human Norovirus Surrogates

Introduction

Human noroviruses are responsible for more than 95% of viral gastroenteritis outbreaks and over 50% of all gastroenteritis outbreaks worldwide (Karst, 2010). However, to date, HuNoVs still cannot be propagated in cell culture or animal models, and the only way to distinguish infectious virus particles from inactivated or noninfectious viruses is human exposure and resulting illness (Richard, 2012). The lack of proper methods for virus culture hinders not

only the studies of the basic virology of HuNoVs but also studies of environmental persistence, routes of transmission, and the inactivation measures of those viruses.

In order to study HuNoVs, the research community turns to viral surrogates. The selection of HuNoV surrogates has been based on the ability of the surrogate to be grown in culture, and its genetic, physical, and/or chemical relatedness to HuNoVs (Richard GP, 2012). Over the years, different surrogates have been used to study various properties of HuNoVs such as the uptake, persistence, distribution and inactivation of virus in food, water and the environment (Cannon et al., 2006). The results of those studies have also been used for developing a better understanding of HuNoVs and improving methods for virus control. In this study, the virus surrogates we used were feline calicivirus and murine norovirus (Type 1). Here, it is important to note that two additional surrogates have been proposed in recent years—porcine sapovirus (a pig calicivirus) (Esseili et al., 2012; Wang et al., 2012) and Tulane virus (a monkey calicivirus) (Farkas et al., 2008; Wei et al., 2008). These surrogates are considered to be promising for the study of HuNoV since both of them are closely related to HuNoVs as well as able to cause gastroenteritis symptoms in animals (DiCaprio et al., 2013).

Feline calicivirus

Feline calicivirus (FCV) was first used as a HuNoV surrogate in late 1990's (Doultree et al., 1999; Slomka et al., 1998) and is currently accepted as a representative surrogate though that is quickly changing with the introduction of new surrogates. This virus belongs to genus *Vesivirus* in the same family with noroviruses, *Caliciviridae*. Although it is primarily a respiratory pathogen in cats and found mainly in respiratory secretions, it is also found in cat feces, which can play a role in FCV spread. In addition, it can also infect dogs where it causes

acute diarrhea (Di Martino et al., 2009). It has been commonly applied in studies involving the evaluation of chemical disinfectants (Satter et al., 2011; Morino et at., 2009) and processing interventions such as heating, freezing and irradiation (Buckow et al., 2008; Butot et al., 2008, 2009; Fino and Kniel 2008). However, several studies have shown that FCV is less stable than other HuNoV surrogates under various conditions such as acidic and alkaline pHs (Cannon et al., 2006), moderate temperatures (50- 60℃) (Gibson and Schwab, 2011), UV irradiation (Park et al, 2011), and chemical treatment by 2% trisodium phosphate (D'Souza and Su, 2010). Those properties of FCV may undermine its usefulness as a HuNoV surrogate.

Murine norovirus Type 1

In 2003, Karst et al. reported and characterized murine norovirus (MNV), the first NoV grown in cell culture with mouse dendritic cell line RAW 264.7. It belongs to NoV GV and is able to cause lethal infections in mice presenting as hepatitis, pneumonia, or inflammation of the nervous system. Though MNV Type 1 (MNV-1) was originally isolated from the brains of severely immunocompromised mice, it is clear now that this virus is an effective enteric pathogen which is shed in mouse feces and is commonly transmitted by the fecal-oral route (Karst et al., 2003).

The most important characteristics of MNV-1 for the analysis of HuNoVs are the commonalities that MNV-1 has with other noroviruses as well as caliciviruses in general. MNV-1 shares many physical features such as size (27-35 nm in diameter) and protein capsid structure (icosahedral) with HuNoVs. In addition, the MNV-1 genome has three ORFs encoding proteins of similar function within two genera in the *Caliciviridae* family, noroviruses and vesiviruses (Karst et al., 2003). Because of the close genetic relationship to HuNoVs, it is considered a better

surrogate for increasing knowledge of HuNoV properties and replication mode when compared to FCV (Ward et al., 2007; Wobus et al., 2004, 2006). Currently, MNV-1 is used frequently in research involving the study of HuNoVs for resistance to chemical disinfections (Hewitt et al, 2009) and processing intervention such as heating (Sow et al, 2011), radiation (Park et al, 2011), electron beam (Sanglay et al. 2011) and high pressure (Lou et al. 2011). To date, MNV-1 is currently the most widely used HuNoV surrogate since it is the only norovirus known to replicate in a cell line and small animal model (Rocha-Pereira et al., 2014)

IV. Free-living Amoeba

Free-living amoebae (FLA) are widespread in nature and are normally found in fresh water microbial ecosystems (Khan, 2006). The term 'amoeba' encompasses a diverse group of organisms in the protozoa, unicellular eukaryote microorganism. Although these organisms share a common amoeboid motion, they have been classified into several different groups.

By feeding on various microorganisms and contributing to nutrient recycling, they are thought to play an important role in biofilm formation by grazing on those biofilm (Pedersen, 1982). Some FLA species are opportunistic pathogens to humans (Visvesvara et al., 2007), but fortunately these types of infections are not very common. Therefore, in general, FLA are not thought to be a major threat to public health. However, it has been repeatedly demonstrated that FLA can interact with various pathogenic microorganisms in ways that may benefit those microorganisms. This relationship between pathogens and FLA has mainly been studied with bacteria but has also been observed with fungi and viruses (Thomas et al., 2010).

V. *Acanthamoebae* spp.

Acanthamoeba is a genus of common FLA. The genus was first discovered in 1930 as eukaryotic cell culture contaminants and then placed in genus *Acanthamoeba* (Castellanii, 1930; Douglus, 1930; Volkonsky, 1931). They are ubiquitous in air, soil, and water environments and can be found in chlorinated swimming pools, drinking water, cooling towers, natural thermal water, hospital water networks, and even marine water. *Acanthamoeba* acts as an opportunistic pathogen to humans and accounts for two well-recognized infections including 1) *Acanthamoeba* keratitis which can lead to blindness (Naginton et al., 1974) and 2) fatal granulomatous amoebic encephalitis (GAE) in immunocompromised populations (Jager and Stamm, 1972; Khan et al., 2006). *Acanthamoeba* keratitis is caused by the use of contact lenses that are exposed to *Acanthamoeba*-contaminated water, and GAE is due to the inability of immunocompromised people to eliminate the pathogen (Khan et al., 2006). In the past twenty years, the number of *Acanthamoeba* infections has risen significantly due to more people wearing contact lenses and an increase in the immunocompromised population (Khan, 2006). In addition to its direct infection in humans, more evidence has been reported that *Acanthamoeba* can interact with other human pathogens such as bacteria and viruses, and thus may play an important role in the ecology and transmission of these pathogens. These interactions will be described further in Section VI (Khan, 2006).

There are two stages of the *Acanthamoeba* life cycle: active trophozoite stage and dormant cyst stage. Trophozoite means “feeding animal” in Latin, and in this stage, *Acanthamoeba* is a round or oval shape with a diameter of 13.5-22 µm and presents spiny cytoplasmic surface structures known as acanthopodia. During the trophozoite stage, *Acanthamoeba* actively feeds on bacteria, yeast, algae and small organic particles by

phagocytosis and replicates by mitosis (Brown and Barker, 1999). Most interactions with other microorganisms happen in this stage. When the living environment becomes unfavorable or adverse to survival such as lack of water, nutrient deprivation, or significant temperature change, trophozoites transform into cysts, which have a round shape and double cell walls with an average diameter of 9-12 µm. The cyst stage is a protective stage because the outer cell wall serves as a shell to help the cyst survive in hostile conditions and resist a variety of physical (dryness, heat, freezing, UV radiation) and chemical (chloride and antimicrobials) agents that can kill trophozoites (Aksozek et al., 2002; Coulon et al., 2010). In this stage, the *Acanthamoeba* is dormant by becoming metabolically inactive and will remain dormant until the environment becomes suitable enough to switch back (e.g., excyst) to the trophozoite stage. The encystment, the transformation of trophozoites into cysts, plays a crucial role in the results of interactions between human pathogens and amoeba.

VI. *Acanthamoeba* and Human Pathogens

Since *Acanthamoeba* are quite prevalent in a variety of environments, they can be found and recovered from similar sources as many human pathogens. Therefore, the interactions between *Acanthamoeba* and those pathogens are an interesting subject to explore. Though the mechanisms of these phenomena are not fully understood, interactions between *Acanthamoeba* and many foodborne pathogens have been studied for decades (Gourabathini et al., 2008; Scheid et al., 2012). To date, this relationship between human pathogens and FLA has been associated with enhanced environmental survival, increased virulence, and increased resistance to biocides and antibiotics in pathogens that undergo intra-protozoal growth (Cirillo et al., 1994; Mattana et al., 2006; Douesnard-Malo and Diagle, 2011).

Bacterial pathogens

The interactions between *Acanthamoeba* and bacteria have been studied since 1954 (Drozanski, 1956) and recent research demonstrates the variability of these interactions (Huws et al., 2008; Snelling et al., 2006). In addition to being the prey of *Acanthamoeba*, many studies have demonstrated the ability of pathogenic bacteria to resist digestion by amoebae, and internalize and even replicate within host amoebae. Moreover, these internalized bacteria can also be released or escape later from the host amoebae (Greub and Raoult, 2004). These interactions between *Acanthamoeba* and bacterial pathogens can depend on the virulence of the amoebae, virulence of the bacteria, and changes in environmental conditions (Khan, 2006). For example, Chekabab et al. (2012) reported that, compared to non-virulent *E. coli* strains, enterohemorrhagic *E. coli* strains were better able to internalize within *Acanthamoeba,* and this ability was dependent on the phosphate (Pho) regulon which plays a key role during bacteria infection, Also, Douesnard-Malo and Daigle (2011) reported that *Salmonella enterica* was able to internalize in amoebae especially when there was a threat to its survival in the environment such as antibiotics. Other foodborne outbreak – related bacteria which have been reported in amoeba-bacteria interactions also include *Listeria monocytogenes, Clostridium perfingens,* and *Campylobacter jejuni* (Zhou et al., 2007; Hadas et al., 2004; Olofsson et al., 2013).

Pathogenic viruses

In contrast to bacteria, only a few pathogenic viruses have been studied for their interactions with *Acanthamoeba*. In 1981, the first study involving the enhanced survival of viruses (i.e., enteroviruses) bound to amoebae was reported by Danes and Cerva. However, no additional research about the interaction or relationship between viruses and FLA has been

published until recently. In 2006, Mattana *et al.* reported that coxsackie virus b3 (a type of human enterovirus) was able to survive within *Acanthamoeba* trophozoites, and infectious virus particles were detected at a significant level after a six month cycle of encystment and excystment. In 2012, a study by Scheid and Schwarzenberger, they demonstrated the intake of adenoviruses into the cytoplasm of *Acanthamoeba* trophozoites and the ability of adenoviruses to remain intact within host amoebae. Besides these three viruses, there are no other reports of well-known pathogenic viruses interacting with or surviving inside FLA.

VII. *Acanthamoeba* as Potential Reservoirs or/and Transportation for Viral Foodborne Pathogens

Interactions between *Acanthamoeba* and foodborne pathogens are quite complex since *Acanthamoeba* also feeds on these microorganisms. According to published findings and reports, the role of *Acanthamoeba* in these interactions may be viewed as a reservoir and/or the transportation mechanism between hosts as well as an adequate environment for foodborne pathogens to replicate (Khan, 2006; Greub and Raoult, 2004; Thomas et al., 2010), though some mechanisms are not yet very clear.

For those bacteria that are able to resist the digestion by amoebae and stay or replicate inside amoebae, the amoebae serve as reservoirs. Amoebae allow those bacteria to be transmitted throughout the environment, evade host defenses or drugs, and reproduce in sufficient numbers to cause disease. Moreover, when in favorable conditions, the increasing bacterial density can lyse their host amoebae and infect new amoebae or move on to cause diseases. On the other hand, the ability of *Acanthamoeba*, especially in cyst stage, to resist harsh conditions such as extreme

temperatures, pH, and osmolarity (Weisman, 1976; Cordingley et al., 1996; Turner et al., 2000;), suggests their potential as being vectors for bacteria and provide transportation and protection for bacteria during transmission (Khan et al., 2006).

To date, there are only three published studies investigating the relationship between viruses and *Acanthamoeba*. These studies reported on the extended survival of poliovirus by binding to amoebae, the intake of adenovirus by amoebae, and the internalization of coxsackie virus b3 within the amoebae while maintain infectivity. Though amoeba phagocytosis (Moliner et al., 2010) and bacteria binding to amoeba via specific receptors (Tyson et al., 2013) may be mechanisms for bacteria entering amoeba, the mechanisms that allow viruses to enter amoeba have not been fully realized. However, those interactions between viruses and *Acanthamoeba* suggests that *Acanthamoe*ba may play the same roles as those in bacteria-amoeba interactions with amoeba-virus interactions.

VIII. Fresh Produce

Fresh produce is recognized as an important source of nutrition, vitamins, and fiber in human daily diet. The consumption of fresh produce has increased over the past two decades because of more awareness of nutrition balance and staying healthy (Warriner et al., 2009). Also, data for the United States shows that the importation of fruits and vegetables in 2004 added up to $12.7 billion—double that reported in 1994 (Aruscavage et al., 2006). On the other hand, possibly related to the increase in consumption, fresh produce is also increasingly recognized as a source of foodborne disease outbreaks. In the United States, the proportion of outbreaks linked to fresh produce increased from 1% in 1970s to 6% in 1990s (Sivapalasingam, 2004). In addition,

CDC also estimates that at least 12% of foodborne disease outbreaks associated with microbial illnesses were linked back to fresh produce items (FDA, 2004). Since most produce grows in the natural environment, receives minimal processing, and is often eaten raw, it is vulnerable to contamination of pathogens and likely to be the risk factor for a foodborne disease outbreak.

Human norovirus outbreak linked to fresh produce

In 2012, Hall et al reported that among HuNoV outbreak, 33% were linked to contaminated leafy greens, moreover, 64, 67, and 47% of outbreaks occurring between 1990-2005 in the U.S. that identified with greens-based salads, fruits, and lettuce, respectively, were attributed to HuNoV (DeWaal and Bhuiya, 2007). In addition, there are several norovirus outbreaks linked back to fresh produce: raspberry cakes using fresh raspberry in south Sweden in 2001 (Le Guyader et al., 2004); lollo bionda lettuce which caused 11 outbreaks in Denmark 2010 (Ethelberg et al., 2010); imported frozen raspberry which caused 13 outbreaks in Finland 2009 (Sarvikivi et al., 2012); and recently, frozen strawberries which caused the largest recorded foodborne outbreak in Germany 2012 (Bernard et al., 2014).

IX. Conclusion

With respect to the prevention and control of HuNoV contamination in the food chain, understanding the microbial ecology, reservoirs, and transmission of HuNoV is one of the major challenges that the food industry faces. The objective of this research revolved around the consideration of *Acanthamoebae* as potential environmental reservoirs or vehicles for transmission of human pathogens combined with fresh produce as food vehicles of norovirus outbreaks caused by HuNoV. In order to investigate this potential connection between FLA and

HuNoV transmission, we aim to evaluate and characterize the interaction between *Acanthamoeba* and HuNoV surrogates to better understand the possible role of *Acanthamoeba* in virus transmission. Furthermore, we propose to investigate the transfer of *Acanthamoeba*, with and without virus association, from water and food preparation surfaces to fresh produce to determine the impact of virus association on amoeba transfer. This study has shown tremendous public health implications by providing novel information about one possible transmission pathway of HuNoV in the environment.

References

Aksozek, A., McClellan, K., Howard, K., Niederkorn, J. Y., & Alizadeh, H. (2002). Resistance of *Acanthamoeba castellanii* cysts to physical, chemical, and radiological conditions. *J Parasitol, 88*(3), 621-623.

Aruscavage, D., Lee, K., Miller, S., LeJeune, J.T., 2006. Interactions affecting the proliferation and control of human pathogens on edible plants. *J. Food Sci. 71*(3), 89-99.

Barker, J., Humphrey, T. J., & Brown, M. W. (1999). Survival of *Escherichia coli* O157 in a soil protozoan: implications for disease. *FEMS Microbiol Lett, 173*(2), 291-295.

Bernard, H., Faber, M., Wilking, H., Haller, S., Höhle, M., Schielke, A., et al. (2014). Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012. *Euro Surveill, 19*(8), 20719.

Buckow, R., Isbarn, S., Knorr, D., Heinz, V., & Lehmacher, A. (2008). Predictive model for inactivation of feline calicivirus, a norovirus surrogate, by heat and high hydrostatic pressure. *Appl Environ Microbiol, 74*(4), 1030-1038.

Bull, R. A., Eden, J. S., Rawlinson, W. D., & White, P. A. (2010). Rapid evolution of pandemic noroviruses of the GII.4 lineage. *PLoS Pathog, 6*(3), e1000831.

Bull, R. A., & White, P. A. (2011). Mechanisms of GII.4 norovirus evolution. *Trends Microbiol, 19*(5), 233-240.

Butot, S., Putallaz, T., Amoroso, R., & Sánchez, G. (2009). Inactivation of enteric viruses in minimally processed berries and herbs. *Appl Environ Microbiol, 75*(12), 4155-4161.

Butot, S., Putallaz, T., & Sánchez, G. (2008). Effects of sanitation, freezing and frozen storage on enteric viruses in berries and herbs. *Int J Food Microbiol, 126*(1-2), 30-35.

Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L. A., & Vinjé, J. (2006). Surrogates for the study of norovirus stability and inactivation in the environment: A comparison of murine norovirus and feline calicivirus. *J Food Prot, 69*(11), 2761-2765.

Castellani A (1930) An amoeba found in culture of yeast: preliminary note. *J Trop Med Hyg* 33: 160.

Centers for Disease Control and Prevention. (2009) CDC Estimates of Foodborne Illness in the United States. http://www.cdc.gov/foodborneburden/estimates-overview.html/ Accessed on: 9/5/2013

Centers for Disease Control and Prevention. (2011). Updated norovirus outbreak management and disease prevention guidelines. *MMWR* 60 (No. RR-3):1-20.

Chekabab, S. M., Daigle, F., Charette, S. J., Dozois, C. M., & Harel, J. (2012). Survival of enterohemorrhagic *Escherichia coli* in the presence of *Acanthamoeba castellanii* and its dependence on Pho regulon. *Microbiologyopen, 1*(4), 427-437.

Cirillo, J. D., Falkow, S., & Tompkins, L. S. (1994). Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infect Immun, 62*(8), 3254-3261.

Cordingley, J. S., Wills, R. A., & Villemez, C. L. (1996). Osmolarity is an independent trigger of *Acanthamoeba castellanii* differentiation. *J Cell Biochem, 61*(2), 167-171.

Coulon, C., Collignon, A., McDonnell, G., & Thomas, V. (2010). Resistance of *Acanthamoeba* cysts to disinfection treatments used in health care settings. *J Clin Microbiol, 48*(8), 2689-2697.

D'Souza, D. H., & Su, X. (2010). Efficacy of chemical treatments against murine norovirus, feline calicivirus, and MS2 bacteriophage. *Foodborne Pathog Dis, 7*(3), 319-326.

Di Martino, B., Di Rocco, C., Ceci, C., & Marsilio, F. (2009). Characterization of a strain of feline calicivirus isolated from a dog faecal sample. *Vet Microbiol, 139*(1-2), 52-57.

Dicaprio, E., Ma, Y., Hughes, J., & Li, J. (2013). Epidemiology, prevention, and control of the number one foodborne illness: human norovirus. *Infect Dis Clin North Am, 27*(3), 651-674.

Douesnard-Malo, F., & Daigle, F. (2011). Increased persistence of *Salmonella enterica* serovar Typhi in the presence of *Acanthamoeba castellanii*. *Appl Environ Microbiol, 77*(21), 7640-7646.

Douglas M (1930) Notes on the classification of the amoeba found by Castellani on culture of a yeast-like fungus. J Trop Med Hyg 33: 258-259.

Doultree, J. C., Druce, J. D., Birch, C. J., Bowden, D. S., & Marshall, J. A. (1999). Inactivation of feline calicivirus, a Norwalk virus surrogate. *J Hosp Infect, 41*(1), 51-57.

Drozanski, W. (1956). Fatal bacterial infection in soil amoebae. *Acta Microbiol Pol, 5*(3-4), 315-317.

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F., & Koopmans, M. (2004). Inactivation of caliciviruses. *Appl Environ Microbiol, 70*(8), 4538-4543.

Esseili, M. A., Wang, Q., Zhang, Z., & Saif, L. J. (2012). Internalization of sapovirus, a surrogate for norovirus, in romaine lettuce and the effect of lettuce latex on virus infectivity. *Appl Environ Microbiol, 78*(17), 6271-6279.

Ethelberg, S., Lisby, M., Bottiger, B., Schultz, A. C., Villif, A., Jensen, T., et al. (2010). Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. *Euro Surveill, 15*(6).

Fankhauser, R. L., Noel, J. S., Monroe, S. S., Ando, T., & Glass, R. I. (1998). Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. *J Infect Dis, 178*(6), 1571-1578.

Farkas, T., Sestak, K., Wei, C., & Jiang, X. (2008). Characterization of a rhesus monkey calicivirus representing a new genus of *Caliciviridae*. *J Virol, 82*(11), 5408-5416.

Food and Drug Administration (FDA), 2004. Produce safety from production to consumption: 2004 action plan to minimize foodborne illness associated with fresh produce consumption. Available at: http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/FruitsVegetablesJuices/ FDAProduceSafetyActivities/ProduceSafetyActionPlan/ucm129487.htm/ Accessed on: 29/10/14.

Fino, V. R., & Kniel, K. E. (2008). UV light inactivation of hepatitis A virus, Aichi virus, and feline calicivirus on strawberries, green onions, and lettuce. *J Food Prot, 71*(5), 908-913.

Gibson, K. E., & Schwab, K. J. (2011). Tangential-flow ultrafiltration with integrated inhibition detection for recovery of surrogates and human pathogens from large-volume source water and finished drinking water. *Appl Environ Microbiol, 77*(1), 385-391.

Glass, R. I., Parashar, U. D., & Estes, M. K. (2009). Norovirus gastroenteritis. *N Engl J Med, 361*(18), 1776-1785.

Gourabathini, P., Brandl, M. T., Redding, K. S., Gunderson, J. H., & Berk, S. G. (2008). Interactions between food-borne pathogens and protozoa isolated from lettuce and spinach. *Appl Environ Microbiol, 74*(8), 2518-2525.

Green, K. Y. 2007. Caliciviridae: The Norovirus. Fields Virology; Lippincott Williaams and Wilkins: Philadephia, PA, USA, 949-979

Greub, G., & Raoult, D. (2004). Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev, 17*(2), 413-433.

Hadas, E., Derda, M., Winiecka-Krusnell, J., & Suek, A. (2004). *Acanthamoeba* spp. as vehicles of pathogenic bacteria. *Acta Parasitologica, 49*(4), 000-000

Hall, A. J., Eisenbart, V. G., Etingüe, A. L., Gould, L. H., Lopman, B. A., & Parashar, U. D. (2012). Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008. *Emerg Infect Dis, 18*(10), 1566-1573.

Hewitt, J., Rivera-Aban, M., & Greening, G. E. (2009). Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies. *J Appl Microbiol, 107*(1), 65-71.

Huws, S. A., Morley, R. J., Jones, M. V., Brown, M. R., & Smith, A. W. (2008). Interactions of some common pathogenic bacteria with *Acanthamoeba polyphaga*. *FEMS Microbiol Lett, 282*(2), 258-265.

Jager, B. V., & Stamm, W. P. (1972). Brain abscesses caused by free-living amoeba probably of the genus *Hartmannella* in a patient with Hodgkin's disease. *Lancet, 2*(7791), 1343-1345.

Johnston, C. P., Qiu, H., Ticehurst, J. R., Dickson, C., Rosenbaum, P., Lawson, P., et al. (2007). Outbreak management and implications of a nosocomial norovirus outbreak. *Clin Infect Dis, 45*(5), 534-540.

Karst, S. M. (2010). Pathogenesis of noroviruses, emerging RNA viruses. *Viruses, 2*(3), 748-781.

Karst, S. M., Wobus, C. E., Lay, M., Davidson, J., & Virgin, H. W. (2003). STAT1-dependent innate immunity to a Norwalk-like virus. *Science, 299*(5612), 1575-1578.

Keswick, B. H., Satterwhite, T. K., Johnson, P. C., DuPont, H. L., Secor, S. L., Bitsura, J. A., et al. (1985). Inactivation of Norwalk virus in drinking water by chlorine. *Appl Environ Microbiol, 50*(2), 261-264.

Khan, N. A. (2006). Acanthamoeba: biology and increasing importance in human health. *FEMS Microbiol Rev, 30*(4), 564-595.

Koo, H. L., Ajami, N., Atmar, R. L., & DuPont, H. L. (2010). Noroviruses: The leading cause of gastroenteritis worldwide. *Discov Med, 10*(50), 61-70.

Le Guyader, F. S., Mittelholzer, C., Haugarreau, L., Hedlund, K. O., Alsterlund, R., Pommepuy, M., et al. (2004). Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. *Int J Food Microbiol, 97*(2), 179-186.

Lopman, B., Gastañaduy, P., Park, G. W., Hall, A. J., Parashar, U. D., & Vinjé, J. (2012). Environmental transmission of norovirus gastroenteritis. *Curr Opin Virol, 2*(1), 96-102.

Lou, F., Neetoo, H., Chen, H., & Li, J. (2011). Inactivation of a human norovirus surrogate by high-pressure processing: effectiveness, mechanism, and potential application in the fresh produce industry. *Appl Environ Microbiol, 77*(5), 1862-1871.

Mattana, A., Serra, C., Mariotti, E., Delogu, G., Fiori, P. L., & Cappuccinelli, P. (2006). *Acanthamoeba castellanii* promotion of in vitro survival and transmission of coxsackie b3 viruses. *Eukaryot Cell, 5*(4), 665-671.

Moliner, C., Fournier, P. E., & Raoult, D. (2010). Genome analysis of microorganisms living in amoebae reveals a melting pot of evolution. *FEMS Microbiol Rev, 34*(3), 281-294.

Morillo, S. G., & Timenetsky, M. C. S. T. (2011). Norovirus: an overview. *Rev Assoc Med Bras, 57*(4), 453-458.

Morino, H., Fukuda, T., Miura, T., Lee, C., Shibata, T., & Sanekata, T. (2009). Inactivation of feline calicivirus, a norovirus surrogate, by chlorine dioxide gas. *Biocontrol Sci, 14*(4), 147-153.

Naginton, J., Watson, P. G., Playfair, T. J., McGill, J., Jones, B. R., & Steele, A. D. (1974). Amoebic infection of the eye. *Lancet, 2*(7896), 1537-1540.

Olofsson, J., Axelsson-Olsson, D., Brudin, L., Olsen, B., & Ellström, P. (2013). *Campylobacter jejuni* actively invades the amoeba *Acanthamoeba polyphaga* and survives within non digestive vacuoles. *PLoS One, 8*(11), e78873.

Park, G. W., Linden, K. G., & Sobsey, M. D. (2011). Inactivation of murine norovirus, feline calicivirus and echovirus 12 as surrogates for human norovirus (NoV) and coliphage (F+) MS2 by ultraviolet light (254 nm) and the effect of cell association on UV inactivation. *Lett Appl Microbiol, 52*(2), 162-167.

Patel, M. M., Hall, A. J., Vinjé, J., & Parashar, U. D. (2009). Noroviruses: a comprehensive review. *J Clin Virol, 44*(1), 1-8.

Patel, M. M., Widdowson, M. A., Glass, R. I., Akazawa, K., Vinjé, J., & Parashar, U. D. (2008). Systematic literature review of role of noroviruses in sporadic gastroenteritis. *Emerg Infect Dis, 14*(8), 1224-1231.

Pedersen, K. (1982). Factors regulating microbial biofilm development in a system with slowly flowing seawater. *Appl Environ Microbiol, 44*(5), 1196-1204.

Richards, G. P. (2012). Critical review of norovirus surrogates in food safety research: rationale for considering volunteer studies. *Food Environ Virol, 4*(1), 6-13.

Rocha-Pereira, J., Neyts, J., & Jochmans, D. (2014). Norovirus: targets and tools in antiviral drug discovery. *Biochem Pharmacol, 91*(1), 1-11.

Sanglay, G. C., Li, J., Uribe, R. M., & Lee, K. (2011). Electron-beam inactivation of a norovirus surrogate in fresh produce and model systems. *J Food Prot, 74*(7), 1155-1160.

Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., et al. (2012). Multiple norovirus outbreaks linked to imported frozen raspberries. *Epidemiol Infect, 140*(2), 260-267.

Sattar, S. A., Ali, M., & Tetro, J. A. (2011). In vivo comparison of two human norovirus surrogates for testing ethanol-based handrubs: the mouse chasing the cat! *PLoS One, 6*(2), e17340.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis, 17*(1), 7-15.

Scheid, P., & Schwarzenberger, R. (2012). *Acanthamoeba* spp. as vehicle and reservoir of adenoviruses. *Parasitol Res, 111*(1), 479-485.

Sivapalasingam, S., Friedman, C. R., Cohen, L., & Tauxe, R. V. (2004). Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *J Food Prot, 67*(10), 2342-2353.

Slomka, M. J., & Appleton, H. (1998). Feline calicivirus as a model system for heat inactivation studies of small round structured viruses in shellfish. *Epidemiol Infect, 121*(2), 401-407.

Snelling, W. J., Moore, J. E., McKenna, J. P., Lecky, D. M., & Dooley, J. S. (2006). Bacterial-protozoa interactions; an update on the role these phenomena play towards human illness. *Microbes Infect, 8*(2), 578-587.

Sow, H., Desbiens, M., Morales-Rayas, R., Ngazoa, S. E., & Jean, J. (2011). Heat inactivation of hepatitis A virus and a norovirus surrogate in soft-shell clams (*Mya arenaria*). *Foodborne Pathog Dis, 8*(3), 387-393.

Teunis, P. F., Moe, C. L., Liu, P., Miller, S. E., Lindesmith, L., Baric, R. S., et al. (2008). Norwalk virus: how infectious is it? *J Med Virol, 80*(8), 1468-1476.

Thomas, V., McDonnell, G., Denyer, S. P., & Maillard, J. Y. (2010). Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. *FEMS Microbiol Rev, 34*(3), 231-259.

Trivedi, T. K., Desai, R., Hall, A. J., Patel, M., Parashar, U. D., & Lopman, B. A. (2012). Clinical characteristics of norovirus-associated deaths: A systematic literature review. *Am J Infect Control*.

Turner, N. A., Russell, A. D., Furr, J. R., & Lloyd, D. (2000). Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii*. *J Antimicrob Chemother, 46*(1), 27-34.

Tyson, J. Y., Pearce, M. M., Vargas, P., Bagchi, S., Mulhern, B. J., & Cianciotto, N. P. (2013). Multiple *Legionella pneumophila* Type II secretion substrates, including a novel protein,

contribute to differential infection of the amoebae *Acanthamoeba castellanii*, *Hartmannella vermiformis,* and *Naegleria lovaniensis*. *Infect Immun, 81*(5), 1399-1410.

Visvesvara, G. S., Moura, H., & Schuster, F. L. (2007). Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol Med Microbiol, 50*(1), 1-26.

Volkonsky M (1931) *Hartmanella castellanii* Douglas, et classification des hartmannelles. *Arch Zool Exp Gen 72*:317–339.

Wang, Q., Zhang, Z., & Saif, L. J. (2012). Stability of and attachment to lettuce by a culturable porcine sapovirus surrogate for human caliciviruses. *Appl Environ Microbiol, 78*(11), 3932-3940.

Ward, V. K., McCormick, C. J., Clarke, I. N., Salim, O., Wobus, C. E., Thackray, L. B., et al. (2007). Recovery of infectious murine norovirus using pol II-driven expression of full-length cDNA. *Proc Natl Acad Sci U S A, 104*(26), 11050-11055.

Warriner, K., Huber, A., Namvar, A., Fan, W., & Dunfield, K. (2009). Recent advances in the microbial safety of fresh fruits and vegetables. *Adv Food Nutr Res, 57*, 155-208.

Wei, C., Farkas, T., Sestak, K., & Jiang, X. (2008). Recovery of infectious virus by transfection of in vitro-generated RNA from tulane calicivirus cDNA. *J Virol, 82*(22), 11429-11436.

Weisman, R. A. (1976). Differentiation in *Acanthamoeba castellanii*. *Annu Rev Microbiol, 30*, 189-219.

Wobus, C. E., Karst, S. M., Thackray, L. B., Chang, K. O., Sosnovtsev, S. V., Belliot, G., et al. (2004). Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *PLoS Biol, 2*(12), e432.

Wobus, C. E., Thackray, L. B., & Virgin, H. W. (2006). Murine norovirus: a model system to study norovirus biology and pathogenesis. *J Virol, 80*(11), 5104-5112.

Zhou, X., Elmose, J., & Call, D. R. (2007). Interactions between the environmental pathogen *Listeria monocytogenes* and a free-living protozoan (*Acanthamoeba castellanii*). *Environ Microbiol, 9*(4), 913-922.

Chapter 2: Investigation of Human Norovirus Surrogate Interactions with *Acanthamoeba* spp.

Investigation of Human Norovirus Surrogate Interactions with *Acanthamoeba* spp.

Tun-Yun Hsueh[a] and Kristen E. Gibson[a, #]

Center for Food Safety, Department of Food Science, Division of Agriculture,

2650 N. Young Ave, Fayetteville, AR 72704

University of Arkansas,

Fayetteville, Arkansas, USA[a].

Corresponding Author: Kristen E. Gibson

2650 N. Young Ave, Fayetteville, AR 72704

University of Arkansas

479-575-6844

keg005@uark.edu

Abstract

Human noroviruses (HuNoV) are the most common cause of foodborne disease outbreaks in the United States. With respect to foodborne norovirus outbreaks, it is usually food handlers that are usually identified as the source of contamination. However, numerous outbreaks have been reported for which the food commodity (in these instances, fresh produce) was contaminated during harvest or post-harvest processing though the vehicle of contamination is unclear. Here, we hypothesize that common free-living amoebae (FLA)—ubiquitous in the environment, known to interact with pathogens, and frequently isolated from fresh produce—may act as reservoirs of HuNoV and facilitate the environmental transmission of HuNoV to fresh produce. To investigate FLA as reservoirs for HuNoV, the interactions between two *Acanthamoeba* species, *A. castellanii* and *A. polyphaga*, as well as two HuNoV surrogates, murine norovirus type 1 (MNV-1) and feline calicivirus (FCV), were evaluated in this study. The results showed that after 1 hour of amoeba-virus incubation at 25℃, 490 and 337 PFU of MNV-1 were recovered from *A. castellanii* and *A. polyphaga*, respectively, while only few or no FCV were detected. In addition, prolonged interaction of MNV-1 with amoeba was investigated for a period of 8 days, and MNV-1 was demonstrated to remain stable at around 200 PFU/ml from day 2 to day 8 after virus inoculation in *A. castellannii*. Moreover, after a complete amoeba life cycle (i.e. encystment and excystment), infectious viruses could still be detected. To determine the location of virus associated with amoeba, immunofluorescence experiments were performed and showed MNV-1 transitioning from the amoeba surface to the inside of amoeba over a 24 hour period. Theses results are significant in understanding the HuNoV survival in the environment and the future control of HuNoV outbreaks where FLA may serve as important reservoirs.

Introduction

Noroviruses (NoV) belong to a highly genetically and antigenically diverse genus *Norovirus* of the *Caliciviridae* family. Noroviruses are divided into five genogroups (GI, GII, GIII, GIV, and GV) according to the genetic similarity of highly conserved areas of their genomes such as the RNA-dependent RNA polymerase and the VP1 protein (Green, 2007) with GI, GII and GIV associated with human diseases. They are important enteric pathogens responsible for at least 50% of all gastroenteritis outbreaks worldwide (Karst, 2010), and partially due to their high infectivity and low infectious dose (18-1,000 virus particles) (Teunis et al., 2008), NoVs are the most common cause of foodborne disease outbreaks in the United States (CDC, 2011).

Transmission of human NoVs (HuNoV) occurs primarily through exposure to contaminated food or water, person-to-person contact, aerosolized particles from vomitus, and contact with contaminated fomites (e.g., door handles, toilets, tables, elevator buttons) (Lopman et al., 2012). Numerous waterborne disease outbreaks have been linked to HuNoV (Hewitt et al., 2007; Lysen et al., 2009; Kvitsand and Fiksdal, 2010), and HuNoV is often detected in surface water (Hernandez-Morga et al., 2009), waste water (Aw and Gin, 2010), and even finished drinking water (Victoria et al., 2010). With respect to foodborne disease outbreaks, the most common food commodity implicated in HuNoV outbreaks is fresh produce, specifically leafy greens (Hall et al., 2012); however, the route of transmission and point of contamination are often unknown. For example, national and international outbreaks involving fresh and frozen raspberries or raspberry products (Pönkä et al, 1999; Falkenhorst et al., 2005) and bagged Lollo Bionda lettuce (Ehtelberg et al., 2010) indicated the likelihood of contamination during harvest, transport or processing of the products. In these instances the source of contamination could be

environmental or possibly from an infected field worker. Another more recent outbreak from 2012 related to frozen strawberries imported by Germany from China caused more than 11,000 cases of acute gastroenteritis in German school children (Bernard et al., 2014). Based on various aspects of fresh produce production—exposure to environmental factors such as soil, compost, and water combined with minimal processing—HuNoVs could be introduced into the supply chain through a variety of routes. In addition, HuNoVs may potentially interact with other microorganisms such as free-living amoeba (FLA) that have been isolated from fresh produce purchased from retail settings that is washed and ready to eat (Rude et al., 1984).

Acanthamoeba is a genus of common FLA (Gourabathini et al., 2008). These amoebae are ubiquitous throughout natural environments (soil, water, and sediments) as well as the built environment including chlorinated swimming pools, drinking water distribution systems, cooling towers, and hospital water networks. There are two stages of the *Acanthamoeba* life cycle: active trophozoite stage and dormant cyst stage. In the trophozoite stage, *Acanthamoeba* actively feeds on bacteria, yeast, algae and small organic particles, replicate by mitosis, and has the ability to transform into cyst when the living environment becomes unfavorable or adverse to survival such as desiccation, nutrient deprivation, or significant temperature change (Weisman, 1976). *Acanthamoeba* cysts can be capable of withstanding hostile conditions and a variety of physical (dryness, heat, freezing, UV radiation) and chemical (chloride and antimicrobials) agents that normally inactivate trophozoites (Weisman, 1976; Cordingley et al., 1996; Turner et al., 2000). *Acanthamoeba* can also act as an opportunistic pathogen to humans and is responsible for two well-recognized diseases including 1) *Acanthamoeba* keratitis which can lead to blindness and 2) fatal granulomatous amoebic encephalitis in immunocompromised populations (Khan, 2006).

In addition to its direct infection in humans, more evidence has been reported that *Acanthamoeba* interacts with other human pathogens including bacteria and viruses. Even though FLA primarily feed on bacteria, many are known to contain microbial endosymbionts, including human pathogens. This relationship between human pathogens and FLA has been associated with enhanced environmental survival, increased virulence, and increased resistance to biocides and antibiotics in pathogens that undergo intra-protozoal growth (Cirillo et al., 1994; Mattana et al., 2006; Douesnard-Malo and Daigle, 2011). Bacteria implicated in foodborne disease outbreaks that have been reported to interact with *Acanthamoeba* include enterohemorrhagic *Escherichia coli* (Chekabab et al., 2012), *Salmonella enterica* subsp. *enterica*, *Listeria monocytogenes, Clostridium botulinum,* and *Campylobacter jejuni* (Douesnard-Malo and Daigle, 2011; Anacarso et al., 2012; Huws et al., 2008). Studies involving *Acanthamoeba* interactions with viruses are much more limited including only poliovirus and echoviruses 4 and 30 (Danes and Cerva, 1981), adenoviruses 11 and 41 (Scheid and Schwarzenberger, 2011), and Coxsackie virus B3 (CVB-3) (Mattana et al., 2006). These amoeba-virus interactions include 1) extended survival (i.e. 21 to at least 75 days depending on the virus type) of viruses by binding to amoeba surface (enteroviruses) 2) internalization of viruses in amoeba cytoplasm (adenoviruses); and 3) internalization and survival of viruses through complete life cycle, including trophozoite and cyst forms, of amoeba (CVB-3). However, to the best of our knowledge, no studies exist on the interaction of FLA with HuNoVs or HuNoV surrogates such as murine norovirus type 1 (MNV-1) and feline calicivirus (FCV).

To investigate whether *Acanthamoeba* could serve as a reservoir for the survival and transmission of HuNoV in the environment, we evaluated the interactions between two species of *Acanthamoeba* (*A. castellanii* and *A. polyphaga*) and two HuNoV surrogates (MNV-1 and

FCV) under reproducible culture conditions. First, amoeba trophozoites were inoculated with virus followed by the analysis of virus titer associated with amoeba to evaluate the interactions between amoebae and viruses. Second, the virus-inoculated trophozoites were induced to encyst and then excyst to evaluate the virus titer associated with different stages of amoeba life cycle. Last, indirect immunofluorescence stain was performed to investigate and determine virus localization in trophozoites.

Material and Methods

Amoeba cultivation

Two species of *Acanthamoeba* available at American Type Culture Collection (ATCC; Manassas, VA) were used here – *A. castellanii* (ATCC 50374) and *A. polyphaga* (ATCC 30871). The axenic cultivation of the two *Acanthamoeba* species was in accordance with ATCC protocols. In brief, trophozoites were cultured with 5 ml peptone-yeast extract-glucose medium (pH 6.5) with additives (0.4 mM $CaCl_2$; 4 mM $MgSO_4$ x $7H_2O$; 2.5 mM Na_2HPO_4 x $7H_2O$; 2.5 mM KH_2PO_4; 0.05 mM $Fe(NH_4)_2(SO_4)_2$ x $6H_2O$; 1g/1L Na Citrate x $2H_2O$) (PYG, ATCC medium 712) in T-25 flasks at 25℃. When the amoebae formed an almost continuous sheet of trophozoites on the bottom surface of the flask (i.e. near peak density), the flask was vigorously agitated and approximately 250µl of the suspended amoebae were transferred to a new tube or flask containing 5 ml of fresh PYG medium.

Virus stock preparation

MNV-1 and FCV were prepared as described previously with modifications (Gibson and Schwab, 2011). Briefly, MNV-1 and FCV (both kindly provided by Dr. Kellogg Schwab at Johns Hopkins School of Public Health, Baltimore, MD) were propagated in monolayers of RAW 267.4 (mouse leukaemic monocyte macrophage, ATCC TIB-71) and CRFK (Crandell Rees feline kidney, also provided by Dr. Kellogg Schwab) cells, respectively. RAW 264.7 cells were cultured in complete Dulbecco modified Eagle medium (DMEM) (MediaTech, Inc., Manassas, VA) containing 10% low endotoxin fetal bovine serum (FBS; Biosera North America, Kansas City, MO), 1% 100× penicillin-streptomycin solution, and 1% HEPES (Sigma-Aldrich, St Louis, MO) at 37℃, 5% CO_2. After reaching 80 to 90% confluent, cells were infected with MNV-1 at a multiplicity of infection (MOI) of 0.05. Feline calicivirus was propagated using CRFK cells cultured in complete minimum essential medium (MEM; MediaTech, Inc.) with 10% low endotoxin FBS (Biosera), 1% 100× penicillin-streptomycin solution and 1% nonessential amino acid (Gibco® , Grand Land, NY) at a MOI of 0.01. Both viruses were extracted from cell lysate after complete cytopathic effect was observed by following the protocol previously reported by Gibson and Schwab (2011). Briefly, the culture medium with cells and viruses were subjected to three freeze-thaw cycles at these temperatures −80°C and 37°C, respectively, followed by centrifugation at 5000 × *g* for 20 min, filtration by 0.01 μm filter and storage at -80℃. To determine the infectious titer of MNV-1 and FCV, the plaque assay reported previously (Gibson and Schwab, 2011) was applied with modifications. Briefly, cells were seeded in 6-well plates and grown to 80 to 90% and 100% confluence, for RAW cells and CRFK cells, respectively, in 2 ml of complete growth medium. Cell monolayers were inoculated with virus stocks for 1 hour at 37℃, and after removal of the inocula, cells were overlaid with 2 ml of overlay medium containing 1.5% low melting point agarose (Invitrogen, Carlsbad, CA), and incubated for 48

hours followed by adding 3% neutral red in phosphate buffered saline *(*PBS) to visualize formed plaques. Plaques were ready to be counted 1 to 3 hours after adding the neutral red solution. Plates with 5 to 50 plaque forming units (PFU) were used to determine the virus titer as PFU per milliliter.

Amoeba inoculation with virus

For amoeba virus inoculation with MNV-1 and FCV, the protocol described by Mattana et al. (2006) was used with modifications. Briefly, experiments were performed in suspension in 50-ml conical polypropylene tubes inoculated each with 3×10^6 amoebae in 1ml DMEM and 1ml of virus stock suspension to give a virus to amoeba ratio of 0.1, 1, or 10. Amoeba controls received the same volume of DMEM. The amoebae-virus suspensions were then incubated for one hour at 25℃ followed by centrifugation (100 ×*g*, 5 min) to pellet amoebic cells. The pellet was subsequently washed with PBS in order to remove unassociated viruses, suspended in 1 ml DMEM containing 5% FBS and 1% 100 × penicillin-streptomycin solution, and maintained at 25℃.

Determine the virus titer associated with amoeba after virus inoculation

To determine the number of infectious virus associated with amoeba after virus inoculation, the methods described by Mattana et al. (2006) were used with modifications. The virus-amoebic trophozoite suspensions described above in "Amoeba inoculation with virus" were used for these experiments. Briefly, after 1 hour of incubation followed by wash and resuspension, these

amoebic suspensions were centrifuged (400 ×*g*, 10 min) in order to recover both cell-free supernatants and pellets. All samples were stored at -80℃ until virus titration was performed using RAW and CRFK cells for MNV-1 and FCV, respectively. Prior to titration, pellets were suspended in 1 ml of DMEM, the trophozoites were disrupted by six freeze-thaw cycles, and cell debris were removed by centrifugation. Virus titration was performed as described previously with cell control (DMEM only), virus positive control (virus only), and amoeba control (amoeba without virus-inoculation). All experiments were performed in duplicate and repeated three times.

Evaluation of amoeba growth after virus inoculation

Here, trophozoites inoculated with virus were washed with sterile PBS, suspended in 2 ml PYG medium at a concentration of 1×10^5 cells per ml, and incubated in 6 well plates at 25℃. Amoebae were counted with a hemocytometer at days 2, 4, 6, 8, 10, and 12 following incubation. Amoebae viability was determined by trypan blue exclusion method. Amoebae morphology was observe with a by Nikon microscope (Nikon instrument Inc, Melville, NY) equipped with a 40× lens objectives.

Determining virus titer associated with amoeba over time

To determine the number of infectious virions associated with amoeba at different time points post-inoculation, the methods described by Mattana et al. (2006) were used with modifications. The virus-inoculated amoebic trophozoites described earlier were also used for these experiments. Briefly, immediately (time zero) and days 1, 2, 4, 6, and 8 after virus incubation,

these amoebic suspensions were centrifuged (400 ×*g*,10 min) in order to collect amoeba pellets. All samples were stored at -80℃ until analyzed to determine virus concentrations. Before titration, pellets were suspended in 1 ml of DMEM, the trophozoites were disrupted by six freeze-thaw cycles, and cell debris was removed by centrifugation. The virus titration was performed as described before with cell control (DMEM only), virus positive control (virus only) and amoeba control. All tests were performed in duplicate and repeated three times.

Evaluation of virus associated with amoeba during encystment and excystment

In these experiments, trophozoites at 2 days post-inoculation as described before were washed, suspended in 3 ml of sterile PBS, and incubated at 25℃ in order to induce their encystment (Khan, 2006). After 2 weeks in cyst form, the trophozoites were recovered. Briefly, the cysts were washed twice in PBS and cultured in PYG medium at 25℃ for 2 to 4 days to induce their differentiation into the trophozoitic form. Both cysts and recovered trophozoites were then lysed and used for virus titration as described in previous sections.

Confirmation of virus localization in amoeba by confocal immunofluorescence

A double indirect immunofluorescence technology was used to determine the location of virus (on amoeba surface or inside amoeba). Briefly, at 0 and 24 h post-inoculation, suspensions of virus inoculated trophozoites (a virus to amoeba ratio of 10) were centrifuged and then resuspended in sterile PBS, seeded into chamber slides, and incubated for 1 h at 25℃. Amoebae then were treated for 1 h with anti-MNV-1 mouse immunoglobulin G (IgG) (Alpha Diagnostic

Intl, Inc., San Antonio, TX) to detect the virus localization on the surface of trophozoites. After 1 h of incubation at 25℃, the samples were washed with PBS, fixed with freezing methanol for 45 min, and incubated with anti-mouse IgG fluoresceinisothiocyanate (FITC)-conjugated antibody (KPL, Gaithersburg, MD). Afterwards, the samples were washed again and exposed to the anti-MNV-1 mouse IgG to detect the virus inside trophozoites followed by incubating with anti-mouse IgG tetramethylrhodamineisothiocyanate (TRITC)-conjugated antibody (KPL). After washing with PBS, samples were mounted and examined with a Nikon Eclipse-600 microscope (Nikon instrument Inc,) equipped with a Bio-Rad Microradiance confocal system.

Statistical Analyses

All experiments were performed in either triplicate or duplicate and repeated at least twice. Data was analyzed by student's *t*-test using SAS version 9.3 and the difference between groups were considered significant when P was <0.05.

Results

Qualitative evaluation of virus-amoeba association

The initial association of HuNoV surrogates with *A. castellanii* and *A. polyphaga* was determined based on 1 hour incubation at 25℃. Results indicated that MNV-1 had some interaction with both amoeba species at all three virus to amoeba ratios while FCV showed no

association with amoeba (data not shown). Based on these results, all remaining experiments were performed with only MNV-1 at a virus to cell ratio of 1.

Amoeba growth evaluation

To study the possible effect of MNV-1 inoculation on the growth of *A. castellanii* and *A. polyphaga*, the growth rate of non-inoculated and virus-inoculated trophozoites as well as the morphology of trophozoites were evaluated. After 12 days of incubation at 25℃, control cultures of *A. castellanii* and *A. polyphaga* produced about 7.1×10^6 and 4.9×10^6 amoeba per ml, respectively. Under the same conditions, cultures of virus-inoculated trophozoites in both *Acanthamoeba* species did not show any significant differences ($P < 0.05$) in growth rate (Figure 1). In addition, trophozoites of both amoebae species showed no difference in morphological features between control and virus-inoculated groups under phase-contrast microscopy examination (Figure 2). Finally, cell viability evaluated by trypan blue was >95% for both amoeba species.

Quantitative evaluation of virus titer associated with amoeba

The number of infectious MNV-1 associated with amoeba over time was determined for both amoebae. The virus titration showed that after 1 hour incubation, 490 and 337 PFU were recovered from *A. castellanii* and *A. polyphaga*, respectively (Figure 3). The input virus value was 6.47 $\log_{10}$ and the total number of recovered virus from *A. castellanii* and *A. polyphaga* was 2.69 and 2.52 $\log_{10}$, respectively. The kinetic study of infectious MNV-1 associated with amoeba

was performed over 8 days. For *A. castellanii*, MNV-1 decreased during the first 48 hours from 2.65 to 2.26 $\log_{10}$; however, from day 4 to day 8 after inoculation, the virus number remained stable at approximately 2.27 $\log_{10}$ (Figure 4A). Conversely, for *A. polyphaga*, MNV-1 steadily decreased after inoculation. After day 4, there was no significant difference between amoeba control and virus-inoculated groups (Figure 4B) while the cell negative control showed no plaques.

Quantitative evaluation of virus titer in different stages of amoeba life cycle

The number of infectious MNV-1 associated with amoeba in different stages of the life cycle was determined for both amoebae (Figure 5). In *A. castellanii*, the infectious virus number associated with cysts was 105 PFU, an 80% decrease compared to the virus concentration immediately after virus inoculation. The virus associated with recovered trophozoites was 65 PFU/ml and was still significantly different ($P < 0.05$) from the amoeba-only group. In *A. polyphaga*, virus number in both cysts and recovered trophozoites showed no difference from the amoeba-only group.

Confocal immunofluorescence studies

To further investigate the location of MNV-1 in *A. castellanii* trophozoites, a double indirect immunofluorescence stain was performed using rabbit IgG anti-MNV-1 capsid protein 1 as the primary antibody and goat-anti-rabbit IgG antibodies conjugated with TRITC or FITC as secondary antibodies. These two different fluorescence labeled secondary antibodies can

distinguish viruses adsorbed on the cell surface from those inside the cell. Compared to the control group (amoeba incubated with DMEM only), trophozoites incubated with MNV-1 immediately after one hour incubation showed FITC signals at the corresponding amoeba position under transparent light observation. This result reflects the location of MNV-1 on the surface of trophozoites (Figure 6B). However, the trophozoites examined 24 hours after virus inoculation showed positive TRITC signals inside the trophozoites along with a corresponding weaker FITC signal on the cell surface.

Discussion

Acanthamoebae are one of the most ubiquitous protozoa in the environment and are particularly widespread in soil and water systems (Khan, 2003; Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004). These protozoa can be highly resistant to sanitizing and disinfecting agents such as chlorine (Seal et al., 1999). Human contact with *Acanthamoebae* is very frequent as antibodies against *Acanthamoeba* could be detected in more than 80% of the human population (Chappell et al., 2001). Human noroviruses are highly contagious and responsible for 21 million gastrointestinal illnesses annually in the United States (CDC, 2011). The majority of HuNoV outbreaks are caused by transmission through food, especially leafy greens, possibly contaminated with viruses via polluted water sources or during preparation (Berger et al., 2010; Tuan et al., 2010); however, the route of HuNoV transmission within the environment is still not clear. Since both HuNoV and *Acanthamoeba* can be found in the same environment, and *Acanthamoebae* have been observed to have interactions with human pathogens (bacteria and viruses), the interactions between *Acanthamoebae* and HuNoV

surrogates were investigated to determine if amoebae could play a role in the environmental transmission of HuNoV.

To verify whether FLA can play a role in transmission of HuNoV by serving as either short- or long-term reservoirs, the interactions between *Acanthamoebae* and HuNoV surrogates were investigated *in vitro* in order to determine 1) the extent of virus adhesion, 2) absorption and internalization by protozoan cells, and 3) the role of virus-amoeba contact in transmission of the virus. Two *Acanthamoeba* species were chosen based on previous studies (reference?). *A. castellanii* is reported to have interactions with three non-enveloped viruses of significance to public health (enterovirus, Coxsackie virus B3 and human adenoviruses) while both *A. castellanii* and *A. polyphaga* play a role in many interactions between *Acanthamoebae* and foodborne bacteria (Anacarso et al., 2012)

Our results on virus-amoeba association demonstrated that after one-hour of virus inoculation, MNV-1, but not FCV, has the ability to adhere to both *Acanthamoeba* species. To our knowledge this is the first study to show this association of amoeba with MNV-1—the first culturable NoV. This is also of interest as MNV-1 is more similar to HuNoV than other Norovirus surrogates commonly utilized (Karst et al., 2003) based on phylogenic analysis of the viral protein and viral genome. The biological and molecular properties MNV-1 shares with other NoV are the most important characteristics for it being a preferred surrogate in analyses of the biology and pathogenesis of HuNoVs (Wobus et al., 2004; Ward et al., 2007; Yunus et al., 2010; Subba-Reddy et al., 2011;Thorne and Goodfellow, 2014). Based on this, the association of MNV-1 observed with *Acanthamoeba* may reflect more accurately the interaction between HuNoV and *Acanthamoeba* than FCV association with amoeba.

Kinetic findings of virus titer in amoeba culture for a period of 8 consecutive days indicates that MNV-1 recovered from amoeba cultures from time zero trophozoites are 2.69 and 2.52 $\log_{10}$ viruses from an initial input 6.47 $\log_{10}$ virus in *A. castellanii* and *A. polyphaga*, respectively. When compared with the virus recovery from amoeba trophozoites in previous studies, coxsackie virus B3 (Mattana et al., 2006) and polioviruses (Danes and Cerva, 1981) were recovered at 50% and 30% of the original virus input, respectively whereas the value of recovered MNV-1 was much less (i.e. less than 1%). However, the constant virus titer detected in *A. castellanii* from day 4 to day 8 implies that MNV-1 can still remain infectious and stable at significant concentrations.

To verify the difference in recovered virus titer from amoeba lysate and virus number observed in immunofluorescence stain, we also analyzed the virus titer in *A. castellanii* amoeba sediment (i.e. cell debris remaining after lysing the pellet) separated from amoeba pellet to determine if there were any unreleased viruses after the freeze-thaw step. The virus titer associated with amoeba sediment was 20 to 60 % less than that of pellet lysate but had a similar trend over the period of 6 days (data not shown). Therefore, the association of virus with cellular debris may account for some of the differences in recovery of virus from amoeba. In addition, the primary antibody used to detect MNV-1 in immunofluorescence stain specifically recognizes VP1 protein of virus capsid. This means the antibody detects all virus particles (or capsid protein) inside or on the surface of amoeba while plaque assay only reflects infectious virus number. Further investigation and analysis of immunofluorescence stain and MNV-1 inactivation will be needed to clarify the difference.

Moreover, the differences in virus persistence between the two amoeba species—MNV-1 titer in *A. polyphaga* kept decreasing over time whereas the virus titer remained stable in *A.*

castellanii—may be due to intrinsic differences in the amoeba species though we were not able to determine specific differences within the scope of this study nor have we been able to identify potential reasons based on review of the published literature. Last, we also observed a few plaque forming units (1 to 5 PFU) in the RAW cell monolayer that was inoculated with amoeba cell lysate control used in the virus titration experiments. Based on the significantly higher number of PFU in plates with amoeba lystate + MNV-1, we are confident that these low numbers of plaques are not due to experimental contamination with MNV-1 since plaques numbers would be expected to be much higher if this were the case. However, it is possible that the plaques observed in the RAW cell monolayer inoculated with the amoeba control group were formed by amoeba-associated viruses such as mimiviruses (Ghigo et al., 2008), which have been reported to infect the RAW 267.4 cells. Further analysis of those plaques using molecular methods such as RT-PCR for detecting MNV-1, mimivirus and other possible microorganisms will be needed to investigate the actual cause of plaques.

To further investigate virus-amoeba interactions in two different amoeba species, a double indirect immunofluorescence (IF) technology was applied to study virus adsorption to and absorption into amoeba as well as the location of virus in amoeba. Initial IF stain showed that one-hour after virus inoculation, MNV-1 was located on the surface of *A. castellanii* trophozoites. However, after 24 hours of amoeba-virus interaction, the IF stain revealed less MNV-1 virus on the amoeba surface and the majority of the MNV-1 virus inside the amoeba. Moreover, compared to FITC signals, TRITC signals indicating internal location of MNV-1 appeared more condensed and aggregated to one spot within the trophozoites. We hypothesize that the MNV-1 entering the amoeba may localize to a vacuole or other intracellular region;

however, further confocal microscopy analyses with higher magnification are needed to confirm this hypothesis.

In addition, our study indicates that MNV-1 can still be detected from virus-inoculated trophozoites of *A. castellanii* after a two week period in the cystic form. Previously, it has been shown that encysted amoebae are resistant to various physical and chemical agents, and cysts have been known to survive for several years (Mazur et al, 1995). Therefore, we suggest that encystment of *A. castellanii* may provide MNV-1 with shelter if the virus has been internalized.

In conclusion, this study demonstrates that *A. castellanii* is able to interact with MNV-1; after adhesion to the surface of amoeba, viruses are be internalized by trophozoites in which they survive and maintain infectivity regardless of amoebae replication or encystment. Further experiments are necessary in order to characterize the molecules on the surface of *A. castellanii* involved with MNV-1 adhesion and the mechanisms of MNV-1 internalization in amoeba trophozoites. Based on the data reported in this study, *A. castellanii* appears to have the potential to carry MNV-1 and play a role as a reservoir for HuNoV in aquatic environments.

References

Anacarso, I., de Niederhäusern, S., Messi, P., Guerrieri, E., Iseppi, R., Sabia, C., et al. (2012). *Acanthamoeba polyphaga*, a potential environmental vector for the transmission of food-borne and opportunistic pathogens. *J Basic Microbiol, 52*(3), 261-268.

Aw, T. G., & Gin, K. Y. (2010). Environmental surveillance and molecular characterization of human enteric viruses in tropical urban wastewaters. *J Appl Microbiol, 109*(2), 716-730.

Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P., et al. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environ Microbiol, 12*(9), 2385-2397.

Chappell, C. L., Wright, J. A., Coletta, M., & Newsome, A. L. (2001). Standardized method of measuring *Acanthamoeba* antibodies in sera from healthy human subjects. *Clin Diagn Lab Immunol, 8*(4), 724-730.

Chekabab, S. M., Daigle, F., Charette, S. J., Dozois, C. M., & Harel, J. (2012). Survival of enterohemorrhagic *Escherichia coli* in the presence of *Acanthamoeba castellanii* and its dependence on Pho regulon. *Microbiologyopen, 1*(4), 427-437.

Cirillo, J. D., Falkow, S., & Tompkins, L. S. (1994). Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infect Immun, 62*(8), 3254-3261.

Cordingley, J. S., Wills, R. A., & Villemez, C. L. (1996). Osmolarity is an independent trigger of *Acanthamoeba castellanii* differentiation. *J Cell Biochem, 61*(2), 167-171.

Danes, L., & Cerva, L. (1981). Survival of polioviruses and echoviruses in *Acanthamoeba castellanii* cultivated in vitro. *J Hyg Epidemiol Microbiol Immunol, 25*(2), 169-174.

Douesnard-Malo, F., & Daigle, F. (2011). Increased persistence o*f Salmonella enterica* serovar Typhi in the presence of *Acanthamoeba castellanii*. *Appl Environ Microbiol, 77*(21), 7640-7646.

Ghigo, E., Kartenbeck, J., Lien, P., Pelkmans, L., Capo, C., Mege, J. L., et al. (2008). Ameobal pathogen mimivirus infects macrophages through phagocytosis. *PLoS Pathog, 4*(6), e1000087.

Gibson, K. E., & Schwab, K. J. (2011). Tangential-flow ultrafiltration with integrated inhibition detection for recovery of surrogates and human pathogens from large-volume source water and finished drinking water. *Appl Environ Microbiol, 77*(1), 385-391.

Gourabathini, P., Brandl, M. T., Redding, K. S., Gunderson, J. H., & Berk, S. G. (2008). Interactions between food-borne pathogens and protozoa isolated from lettuce and spinach. *Appl Environ Microbiol, 74*(8), 2518-2525.

Hall, A. J., Eisenbart, V. G., Etingüe, A. L., Gould, L. H., Lopman, B. A., & Parashar, U. D. (2012). Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008. *Emerg Infect Dis, 18*(10), 1566-1573.

Hernandez-Morga, J., Leon-Felix, J., Peraza-Garay, F., Gil-Salas, B. G., & Chaidez, C. (2009). Detection and characterization of hepatitis A virus and Norovirus in estuarine water samples using ultrafiltration--RT-PCR integrated methods. *J Appl Microbiol, 106*(5), 1579-1590.

Hewitt, J., Bell, D., Simmons, G. C., Rivera-Aban, M., Wolf, S., & Greening, G. E. (2007). Gastroenteritis outbreak caused by waterborne norovirus at a New Zealand ski resort. *Appl Environ Microbiol, 73*(24), 7853-7857.

Huws, S. A., Morley, R. J., Jones, M. V., Brown, M. R., & Smith, A. W. (2008). Interactions of some common pathogenic bacteria with *Acanthamoeba polyphaga*. *FEMS Microbiol Lett, 282*(2), 258-265.

Karst, S. M. (2010). Pathogenesis of noroviruses, emerging RNA viruses. *Viruses, 2*(3), 748-781.

Karst, S. M., Wobus, C. E., Lay, M., Davidson, J., & Virgin, H. W. (2003a). STAT1-dependent innate immunity to a Norwalk-like virus. *Science, 299*(5612), 1575-1578.

Karst, S. M., Wobus, C. E., Lay, M., Davidson, J., & Virgin, H. W. (2003b). STAT1-dependent innate immunity to a Norwalk-like virus. *Science, 299*(5612), 1575-1578.

Khan, N. A. (2003). Pathogenesis of *Acanthamoeba* infections. *Microb Pathog, 34*(6), 277-285.

Khan, N. A. (2006). *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiol Rev, 30*(4), 564-595.

Kvitsand, H. M., & Fiksdal, L. (2010). Waterborne disease in Norway: emphasizing outbreaks in groundwater systems. *Water Sci Technol, 61*(3), 563-571.

Lopman, B., Gastañaduy, P., Park, G. W., Hall, A. J., Parashar, U. D., & Vinjé, J. (2012). Environmental transmission of norovirus gastroenteritis. *Curr Opin Virol, 2*(1), 96-102.

Lysén, M., Thorhagen, M., Brytting, M., Hjertqvist, M., Andersson, Y., & Hedlund, K. O. (2009). Genetic diversity among food-borne and waterborne norovirus strains causing outbreaks in Sweden. *J Clin Microbiol, 47*(8), 2411-2418.

Marciano-Cabral, F., & Cabral, G. (2003). *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev, 16*(2), 273-307.

Mattana, A., Serra, C., Mariotti, E., Delogu, G., Fiori, P. L., & Cappuccinelli, P. (2006). *Acanthamoeba castellanii* promotion of in vitro survival and transmission of coxsackie b3 viruses. *Eukaryot Cell, 5*(4), 665-671.

Mazur, T., Hadaś, E., & Iwanicka, I. (1995). The duration of the cyst stage and the viability and virulence of *Acanthamoeba* isolates. *Trop Med Parasitol, 46*(2), 106-108.

Rude, R. A., Jackson, G. J., Bier, J. W., Sawyer, T. K., & Risty, N. G. (1984). Survey of fresh vegetables for nematodes, amoebae, and *Salmonella*. *J Assoc Off Anal Chem, 67*(3), 613-615.

Scheid, P., & Schwarzenberger, R. (2012). *Acanthamoeba* spp. as vehicle and reservoir of adenoviruses. *Parasitol Res, 111*(1), 479-485.

Schuster, F. L., & Visvesvara, G. S. (2004). Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int J Parasitol, 34*(9), 1001-1027.

Seal, D. V., Kirkness, C. M., Bennett, H. G., Peterson, M., & Group, K. S. (1999). Acanthamoeba keratitis in Scotland: risk factors for contact lens wearers. *Cont Lens Anterior Eye, 22*(2), 58-68.

Subba-Reddy, C. V., Goodfellow, I., & Kao, C. C. (2011). VPg-primed RNA synthesis of norovirus RNA-dependent RNA polymerases by using a novel cell-based assay. *J Virol, 85*(24), 13027-13037.

Teunis, P. F., Moe, C. L., Liu, P., Miller, S. E., Lindesmith, L., Baric, R. S., et al. (2008). Norwalk virus: how infectious is it? *J Med Virol, 80*(8), 1468-1476.

Thorne, L. G., & Goodfellow, I. G. (2014). Norovirus gene expression and replication. *J Gen Virol, 95*(Pt 2), 278-291.

Tuan Zainazor, C., Hidayah, M. S., Chai, L. C., Tunung, R., Ghazali, F. M., & Son, R. (2010). The scenario of norovirus contamination in food and food handlers. *J Microbiol Biotechnol, 20*(2), 229-237.

Turner, N. A., Russell, A. D., Furr, J. R., & Lloyd, D. (2000). Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii*. *J Antimicrob Chemother, 46*(1), 27-34.

Victoria, M., Rigotto, C., Moresco, V., de Abreu Corrêa, A., Kolesnikovas, C., Leite, J. P., et al. (2010). Assessment of norovirus contamination in environmental samples from Florianópolis City, Southern Brazil. *J Appl Microbiol, 109*(1), 231-238.

Visvesvara, G. S., Smith, P. D., Healy, G. R., & Brown, W. R. (1980). An immunofluorescence test to detect serum antibodies to *Giardia lamblia*. *Ann Intern Med, 93*(6), 802-805.

Ward, V. K., McCormick, C. J., Clarke, I. N., Salim, O., Wobus, C. E., Thackray, L. B., et al. (2007). Recovery of infectious murine norovirus using pol II-driven expression of full-length cDNA. *Proc Natl Acad Sci U S A, 104*(26), 11050-11055.

Weisman, R. A. (1976). Differentiation in *Acanthamoeba castellanii*. *Annu Rev Microbiol, 30*, 189-219.

Wobus, C. E., Karst, S. M., Thackray, L. B., Chang, K. O., Sosnovtsev, S. V., Belliot, G., et al. (2004). Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *PLoS Biol, 2*(12), e432.

Yunus, M. A., Chung, L. M., Chaudhry, Y., Bailey, D., & Goodfellow, I. (2010). Development of an optimized RNA-based murine norovirus reverse genetics system. *J Virol Methods, 169*(1), 112-118.

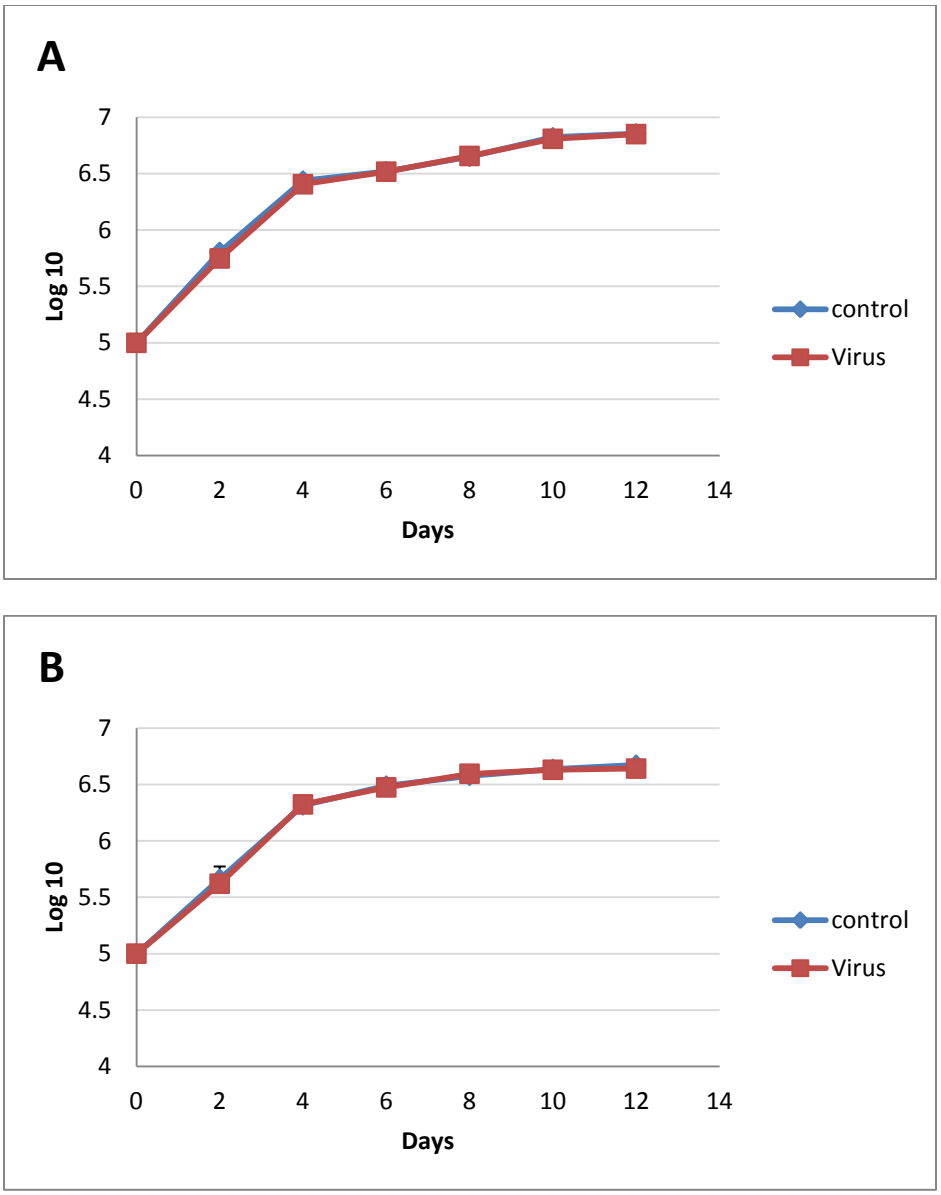

Figure 1. Growth curve of non-inoculated (control) and MNV-1-inoculated (virus) trophozoites evaluated for a period of 12 consecutive days after amoeba-virus inoculation. Amoebae were suspended in 2 ml PYG medium at a density of 1×10^5 cells/ml and at 25°C. In both *A.castellanii* (A) and *A. polyphaga* (B), there was no significant difference between control and virus groups. Values are means ± standard errors from at least three experiments. Significant difference was considered when $P < 0.05$

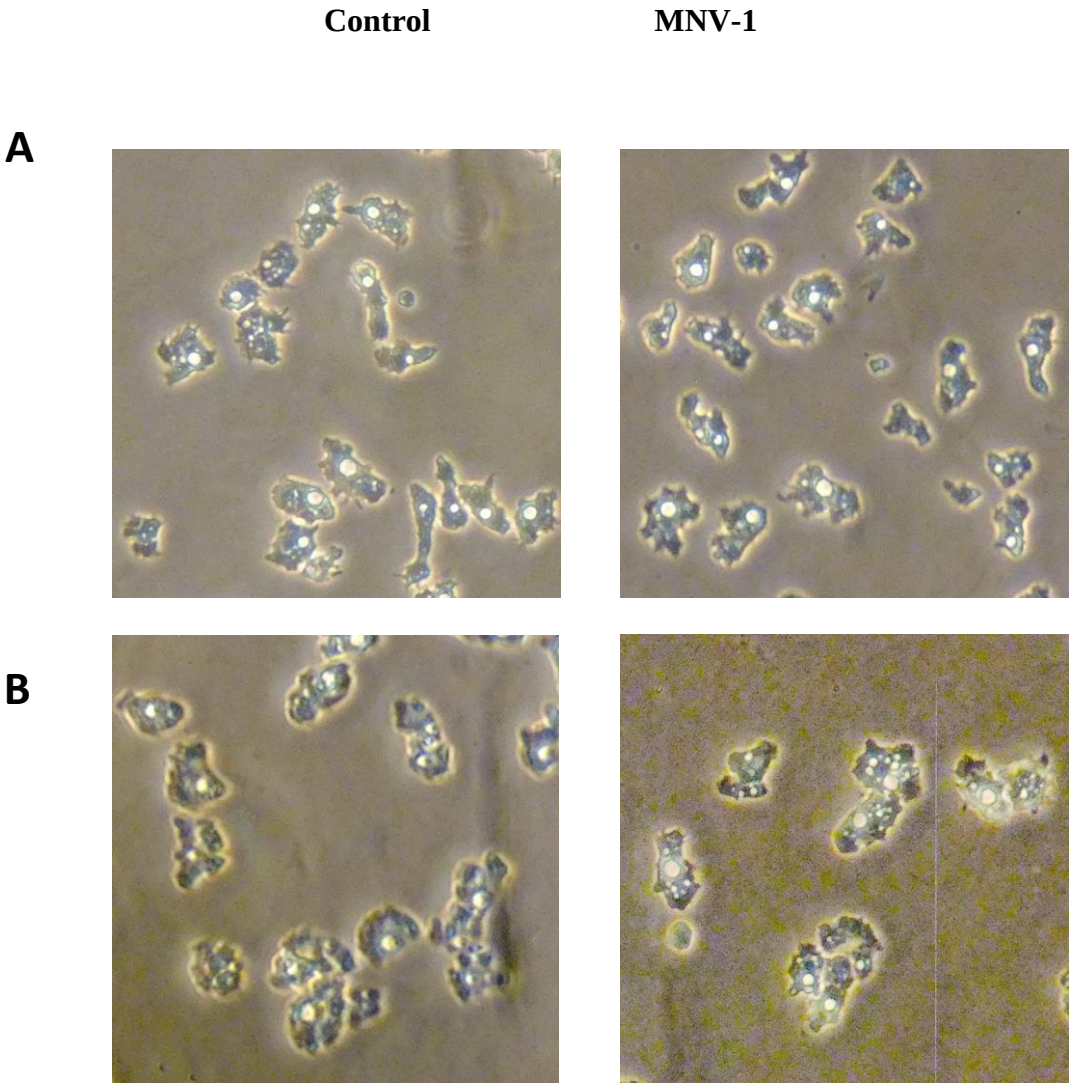

Figure 2. Light micrographs of *Acanthamoeba* trophozites with or without virus inoculation in PYG medium. *A.castellanii* (A) and *A. polyphaga* (B) were inoculated with virus (MNV-1) or DMEM only (Control), and then cultured in PYG medium for 8 days.

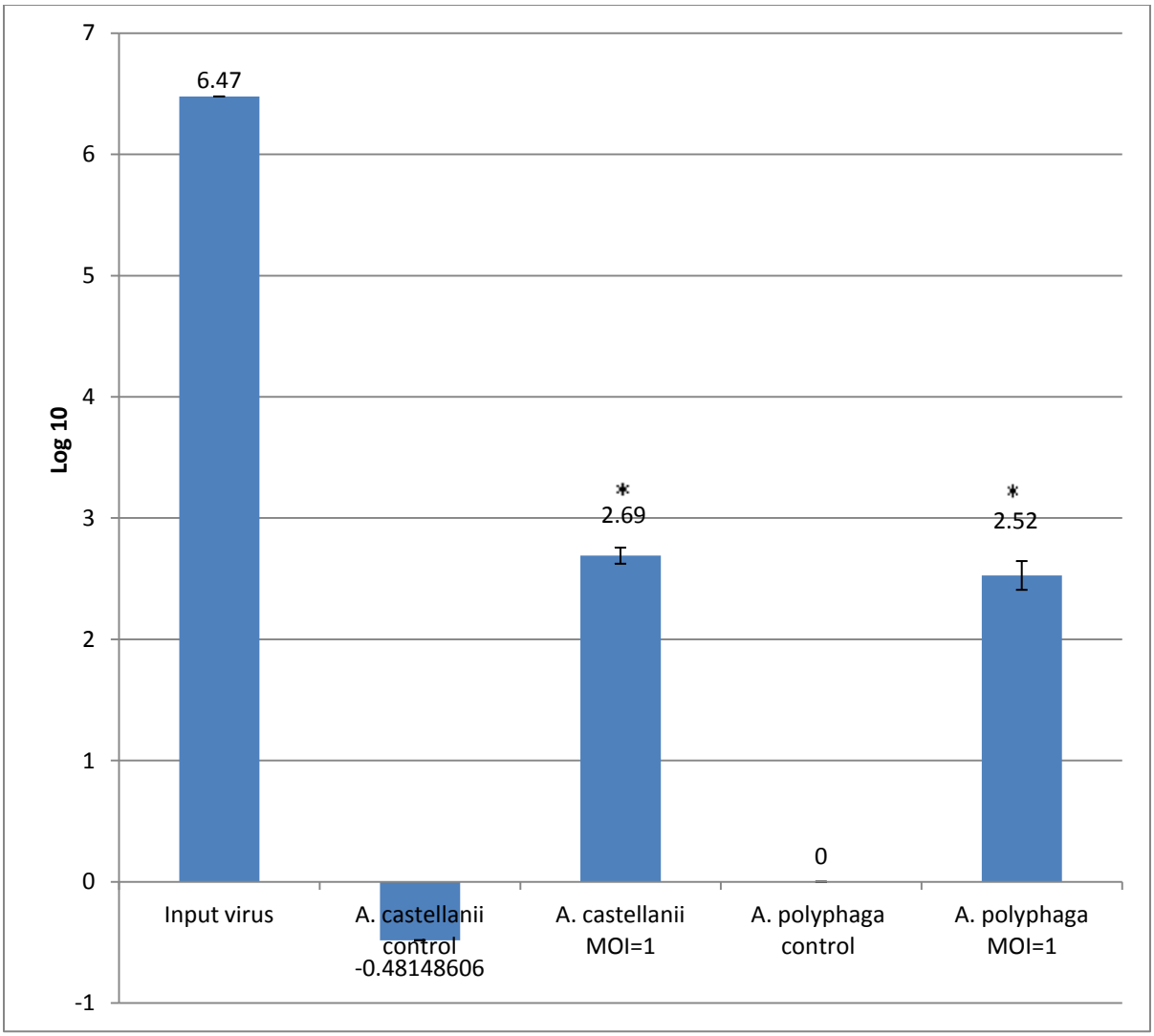

Figure 3. MNV-1 titer associated with *A.castellanii* and *A. polyphaga* after 1 hour virus-amoeba inoculation. The input virus value was $\log_{10}$ 6.47 (3×10^6 PFU) and the recovered virus value mean from *A. castellanii* and *A. polyphaga* was $\log_{10}$ 2.69 (490 PFU) and $\log_{10}$ 2.52 (337 PFU), respectively. Values are means ± standard errors from at least three experiments.*, $P < 0.01$ versus the amoeba control.

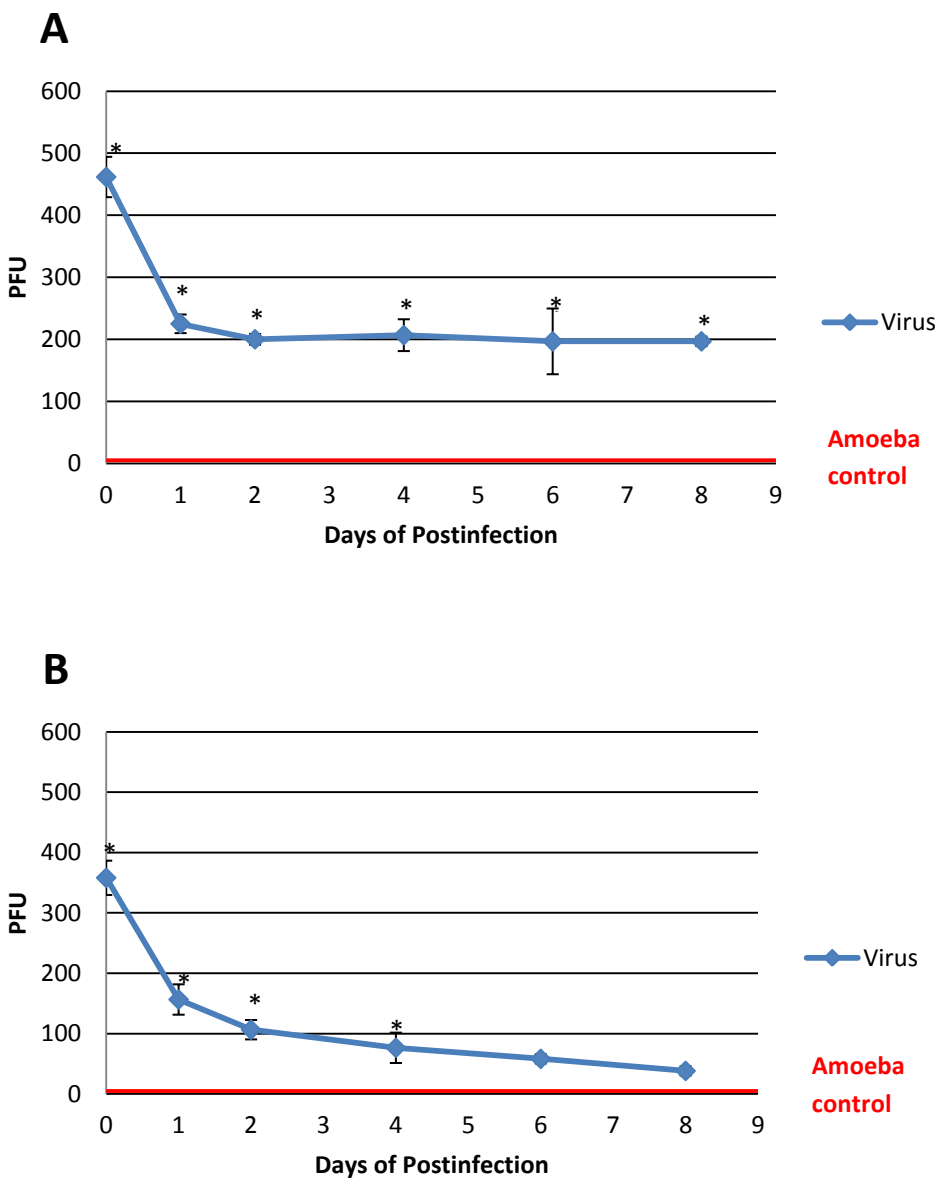

Figure 4. MNV-1 associated with *A.castellanii* (A) and *A. polyphaga* (B) over a period of 8 days after inoculation. Values are means ± standard errors from at least three experiments. Amoeba control group showed the results of amoeba lysate without virus inoculation. *, $P < 0.01$ versus the amoeba control.

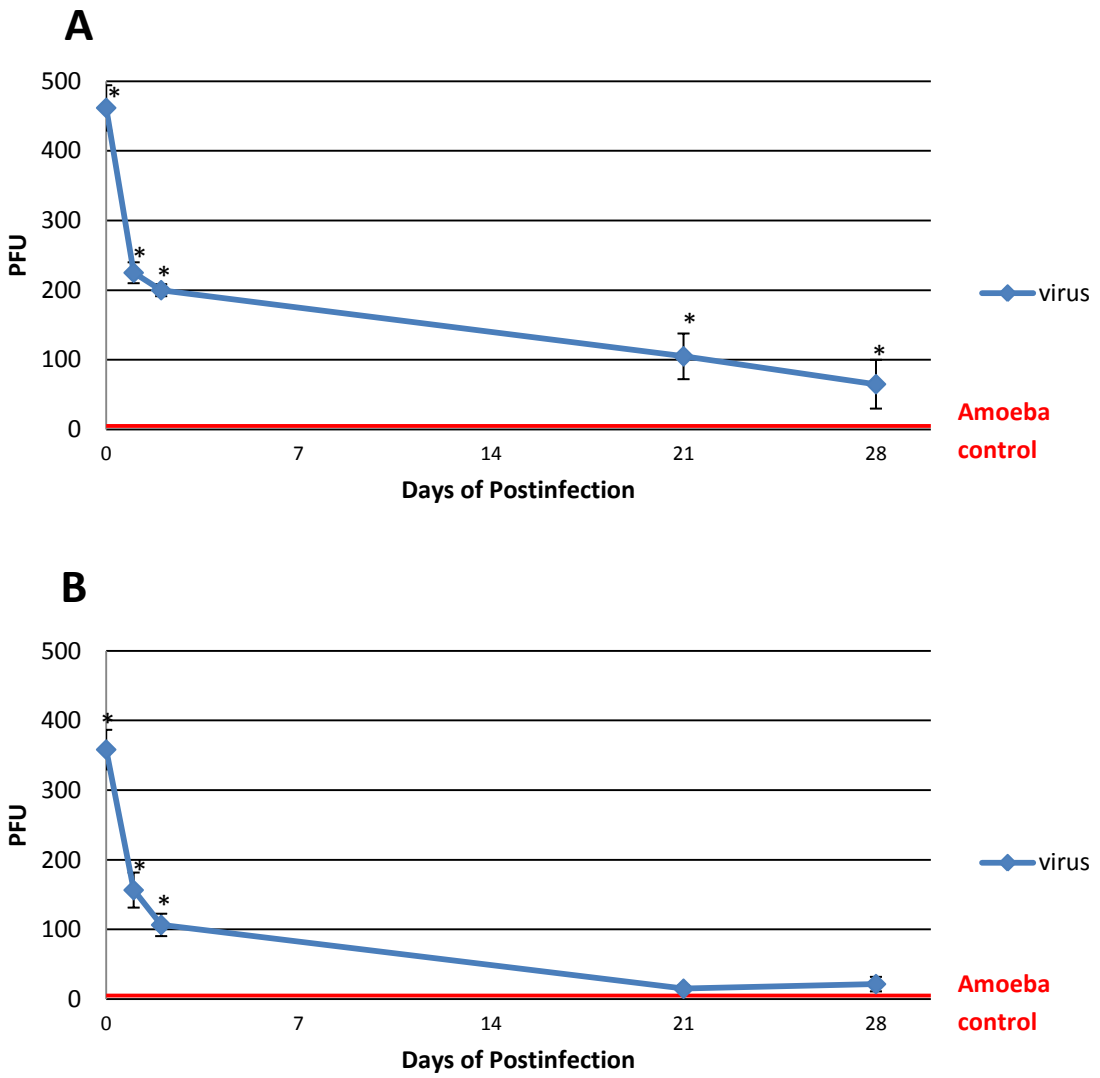

Figure 5. MNV-1 associated with *A. castellanii* (A) and *A. polyphaga* (B) through a complete life cycle of amoeba. Two days after virus inoculation, trophozoites were transferred from PYG medium to PBS to induce encystment. After two weeks in cyst form, the cysts were either collected for virus analysis (day 21) or recovered for trophozoites for another week (day 28) before virus analysis. Values are means ± standard errors from at least three experiments. Amoeba control group showed the results of amoeba lysate without virus inoculation. *, $P < 0.05$ versus the amoeba control.

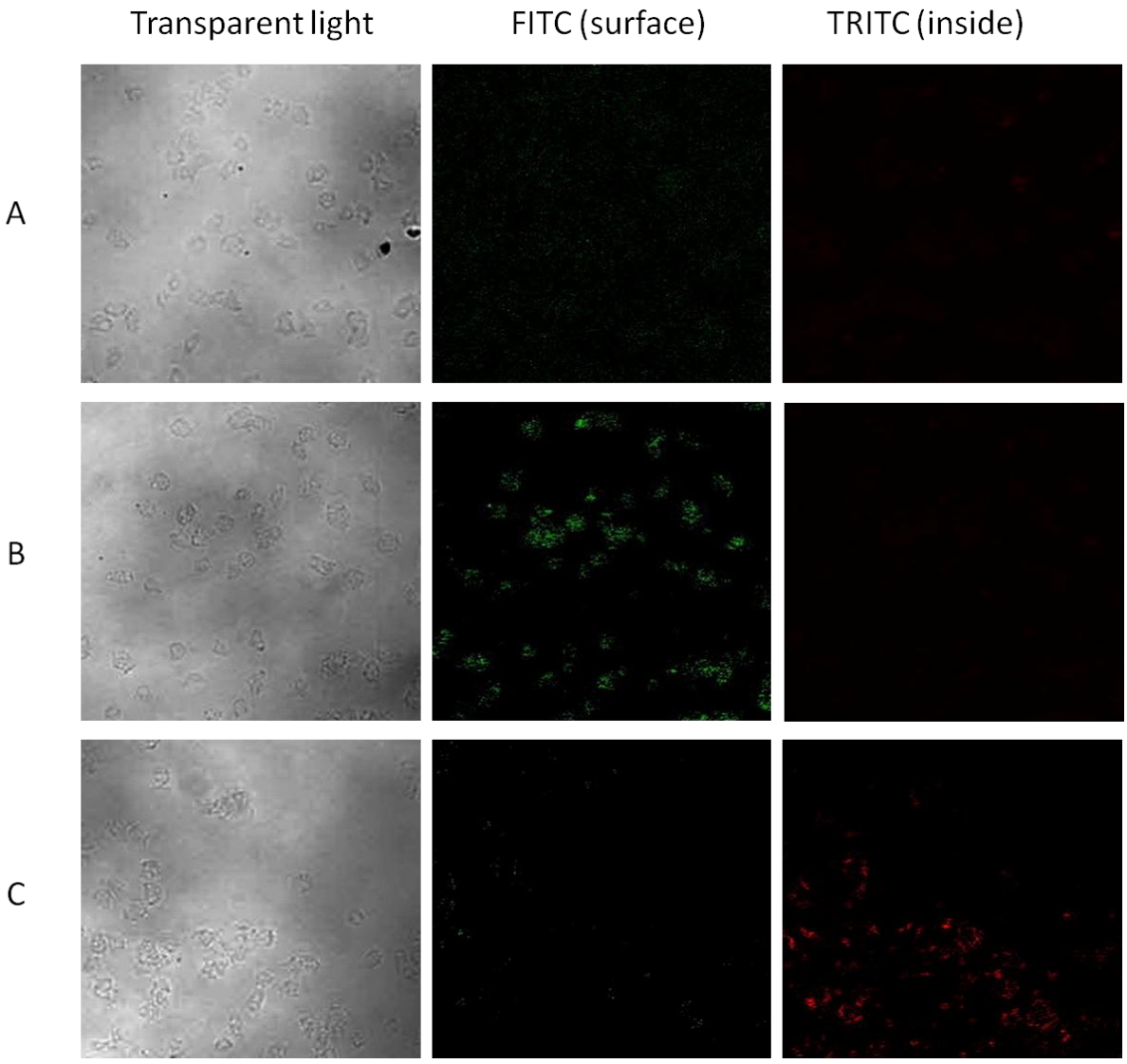

Figure 6. Immunofluorescence pictures of *A. castellanii* trophozoites in DMEM (A) and MNV-1 inoculated *A. castellanii* trophozoites immediately (B) and 24 h (C) after 1 h incubation. Magnification, ×200.

Chapter 3: Investigation of the Transfer of *Acanthamoeba* spp. Associated with Viruses from Water and Environmental Surfaces to Fresh Produce

Investigation of the Transfer of *Acanthamoeba* spp. Associated with Viruses from Water and Environmental Surfaces to Fresh Produce

Tun-Yun Hsueh[a] and Kristen E. Gibson[a, #]

Center for Food Safety, Department of Food Science, Division of Agriculture,

2650 N. Young Ave, Fayetteville, AR 72704

University of Arkansas,

Fayetteville, Arkansas, USA[a].

Corresponding Author: Kristen E. Gibson

2650 N. Young Ave, Fayetteville, AR 72704

University of Arkansas

479-575-6844

keg005@uark.edu

Abstract

Human noroviruses (HuNoV) are the most common cause of foodborne disease outbreaks in the United States. The most frequent commodities implicated in HuNoV outbreaks are leafy greens which can potentially be contaminated during production. With respect to transmission of HuNoV to fresh produce, one hypothesis is that free-living amoebae (i.e. *Acanthamoeba*) that are ubiquitous in the environment (soil, sediments, water) can serve as vehicles of contamination through interaction with viruses—also present in the environment. Here, we investigated the transfer of *Acanthamoeba* spp. associated with murine norovirus—a surrogate for HuNoV—from water and contact surfaces to fresh produce to understand the transfer of amoeba and the effect of virus association with amoeba on the transferability. In water containing a low concentration of amoeba (10^3 cell/ml), the total number of amoeba transferred to 5 g baby kale and spinach was 6.25 to 8.75×10^3 and 2.5 to 3.13×10^3 for 5 cherry tomatoes. Similarly, for high concentrations of amoeba (10^5 cell/ml) in water, the total number of amoeba transferred to baby kale and spinach and cherry tomatoes was between 1.32 to 1.57×10^6 and 5 to 6.65×10^5, respectively. However, as expected, the transfer of amoeba from contact surface to fresh produce was very limited. Overall, the results of this study provide a better understanding of the physical parameters associates with transfer of amoeba to fresh produce, and the potential role that contaminated water (irrigation or wash water) may play in transmission of viruses associated with amoeba.

Introduction

An estimated 9.4 million episodes of foodborne illnesses due to known etiologies occur annually in the United States of which 5.45 million (58%) are caused by human norovirus (HuNoV) (Scallan et al., 2011). Foodborne outbreaks due to HuNoV are commonly caused by food service workers at restaurants, banquet halls, and catered events (Fankhauser et al., 1998). In addition, outbreaks have occurred where the food service workers are not the source. For example, national and international outbreaks involving fresh and frozen raspberries or raspberry products (Pönkä et al, 1999; Le Guyader et al., 2004) and bagged Lollo Bionda lettuce (Ehtelberg et al., 2010) indicated a likelihood of contamination during harvest, transport or processing of the products. In these instances the source of contamination may be environmental or possibly from an infected field worker. Another more recent outbreak from 2012 related to frozen strawberries imported by Germany from China caused more than 11,000 cases of acute gastroenteritis in German school children (Bernard et al., 2014). Due to the widespread nature of these outbreaks along with identical strains within clinical samples, the role of the food service worker is decidedly unlikely.

Food types most commonly associated with HuNoV outbreaks include ready to eat (RTE) foods and in general, foods that are subject to minimal processing. These include but are not limited to bivalve molluscan shellfish (i.e. oysters), fresh produce (i.e. leafy greens, mixed green salads, fruit salads, fruit) deli sandwiches, and baked goods. With respect to fresh produce, 64, 67, and 47% of outbreaks occurring between 1990-2005 in the U.S. that were identified with greens-based salads, fruits, and lettuce, respectively, were attributed to HuNoV—far exceeding the contribution of all bacterial and protozoan foodborne pathogens (DeWaal and Bhuiya, 2007). The most frequent commodities implicated in HuNoV outbreaks among ‘simple’ foods (i.e.

single food ingredient as opposed to deli sandwiches or mixed salads) are reported in Hall *et al.* (2012)—contaminated leafy vegetables contributed to 33% of the outbreaks followed by fruits/nuts (16%) and mollusks (13%). One of the major challenges that the food industry faces with respect to preventing and controlling virus contamination in the food chain is understanding the microbial ecology, reservoirs, and transmission of enteric viruses such as HuNoV. Of interest here is the potential for free-living amoeba (FLA) to serve as environmental reservoirs or vehicles for the transmission of human enteric viruses.

Acanthamoeba is the genus of a common FLA (Visvesvara et al., 1980). These amoebae are ubiquitous throughout natural environments (soil, water, and sediments) as well as the built environment including chlorinated swimming pools, drinking water distribution systems, cooling towers, and hospital water networks. There are two stages of the *Acanthamoeba* life cycle: active trophozoite stage and dormant cyst stage. In trophozoite stage, *Acanthamoeba* actively feeds on bacteria, yeast, algae and small organic particles; replicates by mitosis; and transforms into the cyst stage when the environment becomes unfavorable or adverse to survival such as lack of water, nutrient deprivation, or significant temperature change (Weisman, 1976). Cysts are highly resistant forms of protozoa capable of withstanding hostile conditions and variety of physical (e.g., dryness, heat, freezing, UV radiation) and chemical agents (e.g., sodium hypochlorite, antimicrobials) that normally inactivate trophozoites (Cordingley et al., 1996, Tuner et al., 2000). *Acanthamoeba* can also act as an opportunistic pathogen to humans and is responsible for two well-recognized diseases including 1) *Acanthamoeba* keratitis, which can lead to blindness and 2) fatal granulomatous amoebic encephalitis in immunocompromised populations (Khan et al., 2006).

In addition to its direct infection of humans, more evidence has been reported that *Acanthamoeba* interacts with other human pathogens including bacteria and viruses. Even though FLA primarily feed on bacteria, many human pathogens are able to internalize within amoeba without being digested. Bacteria related to foodborne disease outbreaks that have been reported to interact with *Acanthamoeba* include enterohemorrhagic *Escherichia coli* (Chekabab et al., 2012), *Salmonella enterica* subsp. *enterica*, *Listeria monocytogenes, Clostridium botulinum,* and *Campylobacter jejuni* (Douesnard-Malo and Daigle, 2011; Anacarso et al., 2012; Huws et al., 2008). Pathogens that undergo intra-amoebal growth can have increased environmental survival, possess increased virulence, and increased resistance to biocide and antibiotics (Cirillo et al., 1994; Mattana et al., 2006; Douesnard-Malo and Daigle, 2011). Studies involving *Acanthamoeba* interactions with viruses include enteroviruses (Danes and Cerva, 1981), adenoviruses 11 and 41(Scheid and Schwarzenberger, 2012), and Coxsackie virus B3 (CVB-3) (Mattana et al., 2006). These amoeba-virus interactions could result in extended survival of viruses by binding to amoeba surface (polioviruses); internalization of viruses in amoeba cytoplasm (adenoviruses); and internalization and survival of viruses through complete life cycle, including trophozoite and cyst forms, of amoeba (CVB-3). Moreover, our recent studies also show that *Acanthamoeba* could carry infective murine norovirus type 1(MNV-1)—one of the surrogates for the study of HuNoV—through a complete life cycle and could be a potential vehicle for virus transmission (Chapter 2).

Considering the ubiquity of FLA—specifically *Acanthamoeba*— in the environment and their established relationship with foodborne pathogens, it is possible that *Acanthamoeba* harboring pathogens could enter the food supply at the point of production, harvest, or processing—more specifically, the fresh produce production supply chain. For instance, Rude et

al. (1984) reported the prevalence of *Salmonella*, FLA, and nematodes on fresh vegetables from wholesale and retail sources and found that 30 to 50% of samples were positive for FLA—primarily *A. polyphaga*, *A. rhysodes*, and *A. castellanii*. Although Rude *et al.* (1984) did not investigate the interaction between FLA and pathogens on the fresh produce, a more recent study by Gourabathini *et al.* (2008) made this connection. In Gourabathini *et al.* (2008), FLA isolated from spinach and romaine lettuce were seeded on to cilantro leaves along with *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* Thompson, and the authors reported the sequestration of bacteria by the FLA.

There are two primary ways in which *Acanthamoeba* (and *Acanthamoeba* associated viruses) could possibly contaminate fresh produce: 1) in the field prior to harvest and 2) transfer from packinghouse surfaces or wash water. Here, we hypothesize that fresh produce can become contaminated with *Acanthamoeba* after coming into contact with wash water or food processing surfaces contaminated with *Acanthamoeba*. Therefore, we investigated the ability of *Acanthamoeba* to transfer from water and contact surfaces to fresh produce (baby spinach and kale and cherry tomatoes). Moreover, we evaluated the transfer of amoeba associated with MNV-1 inoculation to determine if this interaction impacted the transfer of amoeba to the fresh produce.

Material and Methods

Fresh produce samples

Five grams baby kale and spinach (approximate 5-8 leaves) or 5 cherry tomatoes were used as fresh produce samples. Both baby kale and spinach (NewStar Fresh Food, LLC, Salinas,

CA), and cherry tomatoes (NatureSweet, San Antonio, TX) were purchased at a national grocery store chain in Northwest Arkansas. Baby kale and spinach were sold as a prewashed and ready to eat product. For each experiment, five grams of baby kale and spinach was taken directly from the package. For the experiments with tomatoes, five cherry tomatoes were first rinsed with dechlorinated tap water (described in method of transmission of *Acanthamoeba* in water to fresh produce experiment**)** to remove dirt on the surface. .

Amoeba cultivation

Two species of *Acanthamoeba* available at American Type Culture Collection (ATCC; Manassas, VA) were used here – *A. castellanii* (ATCC 50374) and *A. polyphaga* (ATCC 30871). The cultivation of two *Acanthamoeba* was in accordance with ATCC protocols. In brief, trophozoites were cultured with peptone-yeast extract-glucose medium (pH 6.5) with additives (0.4 mM $CaCl_2$; 4 mM $MgSO_4$ x $7H_2O$; 2.5 mM Na_2HPO_4 x $7H_2O$; 2.5 mM KH_2PO_4; 0.05 mM $Fe(NH_4)_2(SO_4)_2$ x $6H_2O$; 1g/1L Na Citrate x $2H_2O$) (PYG, ATCC medium 712) in T-25 tissue culture flasks at 25℃. When the amoebic trophozoites reached near 100% confluency on the bottom surface of the flask, the flask was vigorously agitated and 250µl of the suspended amoebae was transferred to a T25 flask containing 5 ml of fresh PYG medium.

Virus stock preparation

MNV-1 was prepared as described previously with modifications (Gibson and Schwab, 2011). Briefly, MNV-1 (kindly provided by Dr. Kellogg Schwab at Johns Hopkins School of

Public Health, Baltimore, MD) were propagated in monolayers of RAW 267.4 (ATCC TIB-71). RAW 264.7 cells were cultured in complete Dulbecco modified Eagle medium (DMEM) (MediaTech, Inc., Manassas, VA) containing 10% low endotoxin fetal bovine serum (FBS; Biosera North America, Kansas City, MO), 1% 100× penicillin-streptomycin solution, and 1% HEPES (Sigma-Aldrich, St Louis, MO) at 37℃, 5% CO_2. After reaching 80 to 90% confluent, cells were infected with MNV-1 at a multiplicity of infection (MOI) of 0.05. Viruses were extracted from cell lysate after complete cytopathic effect was observed by following the protocol previously reported by Gibson and Schwab (2011). To determine the infectious titer of MNV-1, the plaque assay reported previously (Gibson et al., 2012) was applied with modifications. Briefly, RAW 267.4 cells were seeded in 6-well plates and grown to 80 to 90% confluence in 2 ml of complete growth medium. Cell monolayers were inoculated with virus stocks for 1 hour at 37℃, and after removal of the inocula, cells were overlaid with 2 ml of culture medium containing 1.5% low melting point agarose (Invitrogen, Carlsbad, CA), and incubated for 48 hours followed by adding 3% neutral red in phosphate buffered saline (PBS) to visualize formed plaques. Plaques were ready to be counted 1 to 3 hours after adding the neutral red solution. Plates with 5 to 50 plaques are used to determine the virus titer in plaque forming units (PFU) per milliliter.

Preparation of virus-inoculated amoeba

For amoeba virus infection with MNV-1, the protocol described by Mattana et al. (2006) was used with modifications. Briefly, experiments were performed in suspension in 50-ml conical polypropylene tubes inoculated each with 10^6 amoebae in 1ml DMEM and 1ml of virus

stock suspension to give a MOI of 1. Amoeba controls received the same volume of DMEM without viruses. The amoebae-virus suspensions were then incubated for one hour at 25℃ followed by centrifugation (100 × *g*, 5 min) to pellet amoebic cells. The pellet was then washed three times with PBS in order to remove unassociated viruses, suspended in PBS, and maintained at 25℃ for further experiments.

Transmission of *Acanthamoeba* in water to fresh produce

For transfer of *Acanthamoeba* in water to fresh produce samples, experiments were conducted with both ATCC *Acanthamoeba* spp. Briefly, to prepare dechlorinated tap water (DTW), 500 µl of 0.2 M sodium thiosulfate was added into 500 ml of tap water and mixed well. And then, 500 ml of the prepared DTW in a polypropylene container was inoculated with high concentrations (10^5 cells/ml) and low concentrations (10^3 cells/ml) of *Acanthamoeba* with or without virus inoculation. The inoculated water was agitated by gently stirring using a stirring rod for 2 min, and 2 ml of sample was removed to determine the initial concentration of amoeba. Next, 5 g of baby spinach and kale or five cherry tomatoes (prepared as described previously) were added to the water containing amoeba and gently agitated by stirring for 2 with a stir rod and then allowed to equilibrate for 20 min. Samples were removed from amoeba-inoculated water and placed in a whirl-pak bag containing 50 ml of PBS. The bags containing the sample and PBS were shaken for 2 min, and the washings were poured off and collected. Another 50 ml PBS was then added into the bags, and after shaking, the second 50 ml washings were combined with the first one to make the washings a total volume of approximately 100 ml. These washings were then centrifuged at 2,000 × *g* for 20 min, and the pellets were resuspended in 500 µl PBS.

For the experimental control, DTW without amoeba was used. For the sample matrix control, the fresh produce samples were directly placed in the whirl-pak bag containing PBS. The number of amoebae in the suspension was then determined by using trypan blue (Sigma-Aldrich) and a hemocytometer cell counting chamber.

Transmission of *Acanthamoeba* from food contact surfaces to fresh produce

For transfer of *Acanthamoeba* from contaminated surfaces to fresh produce, experiments were conducted with both ATCC *Acanthamoeba* spp. For the experiments, stainless steel sheets (type 304/16 gauge, unpolished; Rose Metal Products, Springfield, MO) and food-grade conveyor belt material (PVC 120 white cover by mini skim coat; Apache, Inc., Grand Rapids, IA) were cut into 4 × 4 in. (10.16 cm^2) coupons. Stainless steel coupons were washed with detergent and autoclaved at 121℃ and 15 psi to sterilize; conveyor belt coupons were prepared by washing with detergent, disinfecting with 70% ethanol followed by a 10% bleach (sodium hypochlorite) solution, rinsing with autoclaved deionized water to remove bleach and then air dried in a laminar flow hood. For each amoeba species, both stainless steel and conveyor belt coupons were inoculated with either high or low levels of amoeba. Five hundred microliters of PBS containing 10^5 (high) or 10^3 (low) amoebae without virus inoculation was spread evenly on each coupon while leaving a 1-in. (2.54 cm) margin and amoebae were allowed to attach to the surface for 15 min at 25C°. After 15 minutes (the coupons were still wet and the amoeba suspension was not dried out), produce samples prepared as described previously were then placed on the coupons and allowed to contact the coupons for 20 min. Fresh produce samples were then removed from the coupons, and total amoeba were determined as described above for

transfer of amoeba from water to fresh produce. For the experimental control, the sterile coupons without amoeba were used.

Analysis, assessment, and interpretation

All experiments were performed in duplicate and repeated at least twice. The arithmetic means of the results, recorded as $\log_{10}$ amoebae, were plotted against produce type and transfer medium (i.e. water or surface), and standard deviations were reported as error bars. These data were analyzed statistically by Student's *t*-test using SAS version 9.3. Results were considered significantly different at $p \leq 0.05$.

Results

Amoeba-virus inoculation

Based on our previous studies, after virus inoculation, the number of MNV-1 recovered from 3×10^6 *Acanthamoeba* was about 2.5 $\log_{10}$ (Chapter 2). In addition, the virus inoculation did not affect morphology under microscopy nor the normal growth of both *Acanthamoeba* species (data not shown).

Transmission of *Acanthamoeba* from water to fresh produce

In 500 ml water containing 10^3 amoeba per ml (low concentration), the total amoeba recovered from baby kale and spinach was 8.75×10^3 (3.94 $\log_{10}$) for both *A. castellanii* and *A.*

polyphaga, and from cherry tomatoes, the total amoeba recovered was 3.13×10^3 ($3.5 \log_{10}$)and 2.5×10^3 ($3.4 \log_{10}$) of *A. castellanii* and *A. polyphaga*, respectively. In 500 ml water containing 10^5 amoeba per ml (high concentration), the total amoeba eluted from baby kale and spinach were 1.49×10^6 ($6.17 \log_{10}$) of *A. castellanii* and 1.32×10^6 ($6.12 \log_{10}$) of *A. polyphaga* while the total amoeba eluted from cherry tomatoes was 6.13×10^5 ($5.79 \log_{10}$) and 6.65×10^5 (5.82 $\log_{10}$) of *A. castellanii* and *A. polyphaga*, respectively. In addition, the results were similar for water containing virus-inoculated amoeba. In water containing low concentrations of virus-inoculated amoeba, the amoeba number eluted from combination of baby kale and spinach and *A. castellanii*, baby kale and spinach and *A. polyphaga*, cherry tomatoes and *A. castellanii*, and cherry tomatoes and *A. polyphaga* were 8.75×10^3 ($3.94 \log_{10}$), 6.25×10^3($3.8 \log_{10}$), 2.5×10^3($3.4 \log_{10}$) and 2.5×10^3 ($3.4 \log_{10}$), respectively (Figure 1A). Also, the result number of amoeba eluted from water containing a high concentration of virus-inoculated amoeba from combinations of baby kale and spinach and *A. castellanii*, baby kale and spinach and *A. polyphaga*, cherry tomatoes and *A. castellanii*, and cherry tomatoes and *A. polyphaga* were 1.33 $\times 10^6$ ($6.12 \log_{10}$) 1.57×10^6 ($6.2 \log_{10}$), 5×10^5 ($5.7 \log_{10}$), and 6.45×10^5 ($5.81 \log_{10}$), respectively (Figure 1B). No amoebae were observed from the sample matrix controls and the experimental control groups. There were no significant differences in the total amoeba eluted between the two *Acanthamoeba* species from either baby kale and spinach or cherry tomatoes. Moreover, the results of water containing virus-inoculated amoeba was not significantly different from those of water containing amoeba without virus inoculation in both *Acanthamoeb*a species.

Transmission of *Acanthamoeba* from food contact surfaces to fresh produce

In the experiments with coupons containing low concentration amoeba (10^3 cells per coupon), no amoebae were observed in any of the fresh produce – *Acanthamoeba* – coupon material combinations. For coupons containing high concentrations of amoeba (10^5 cells per coupon), the total amoeba eluted from all experimental combinations was less than 4 and not significantly different from control groups. No amoebae were observed in control groups.

Discussion

Acanthamoebae are one of the most ubiquitous protozoa in the environment and are widespread in soil and water systems (Khan, 2003; Marciano-Cabral & Cabral, 2003; Schuster &Visvesvara, 2004). These protozoa can also be highly resistant to sanitizing and disinfecting agents such as chlorine (Seal et al., 1999). Human contact with *Acanthamoebae* is considered to be quite frequent as it has been reported in studies conducted in both United States and New Zealand that *Acanthamoebae* antibodies could be detected in more than 80% of the human population (Cursons et al., 1980; Chappell et al., 2001). In this study, we investigated the ability of two species of *Acanthamoeba* (with and without virus association) to transfer from both an aquatic environment and food contact surface to fresh produce. Baby leafy greens and cherry tomatoes were used to present typical examples of fresh produce consumed in United States. In addition, we used dechlorinated tap water instead of de-ionized water in order to avoid extra stress to amoebae and to more closely simulate a real-world scenario.

The amoeba number eluted from baby kale and spinach was significantly higher than that from cherry tomatoes in water. This may be due to the larger surface area of five grams of baby kale and spinach compared to 5 cherry tomatoes resulting in an increased contact surface for

amoeba in water. In addition, the difference in surface texture might also play a role in higher amoeba number in baby leaf samples when compared to tomatoes. The results were not significantly different between two amoeba species and between normal amoeba and virus-inoculated amoeba. Overall, these results were consistent with our previous study that MNV-1 inoculation did not affect the morphology and normal growth of amoeba (Chapter 2)

In amoeba transmission from water to fresh produce experiments, the number of amoeba in high concentration water was 100 fold of that in water with low concentration amoeba and the eluded amoeba number from high concentration groups were approximately 200 fold of that from low concentration group in both baby kale and spinach and cherry tomatoes. Although the concentration we used in our study is much higher than amoeba concentration in the environment (less than 1 amoeba per ml in tap water and about 330 amoeba per ml in dental unit water, Barbeau and Buhler, 2001) the results implied that the transfer efficiency might be better in water containing high concentrations of amoeba than in that containing low concentrations of amoeba.

Biofilms formed by bacteria and other microorganisms can be found on almost all surfaces in aquatic environments (Carris and Sime-Ngando, 2009). By feeding on bacteria *Acanthamoeba* are also able to graze on biofilm material and play a pivotal role in biofilm formation (Huw et al., 2005). In addition to providing nutrients and facilitating amoeba proliferation, biofilms may also enhance *Acanthamoeba* persistence in/on some cleaning agents (most notably contact lens cleaning solution with respect to human illnesses related to amoeba) and increase *Acanthamoeba* binding to the surfaces (Beattie et al., 2003). Based on the ecology of *Acanthamoeba* and its interaction with microorganisms in aquatic environments, we hypothesize that it is possible for *Acanthamoeba* to come into contact with fresh produce via soil,

irrigation water, or even wash water in the packing house thus transferring from these potential environmental reservoirs to fresh produce.

In the real world, the aquatic environment in which *Acanthamoeba* exists is very complicated. For example, in drinking water distribution systems, amoeba can thrive in these environments with chemical agents such as chlorine while feeding on bacteria and grazing on biofilms and interacting with pathogens including *Legionella pneumophila* and *E. coli*—both of which can be internalized inside amoeba, (Huws et al., 2005). However, the experiments described in the present study were conducted in a controlled environment with monocultures of amoebae and a single type of virus when in reality this environment is much more complex and some factors or interactions between microorganisms in aquatic environment could not feasibly be accounted for (Barker and Brown, 1994). Regardless, the results of amoeba transfer from water to fresh produce are still significant while the transfer of amoeba from contact surface was not significant and might be neglected in further more complex experimental set-ups.

Overall, this is the first study investigating and observing the transfer of *Acanthamoeba* from the environment to fresh produce. These results indicate that the transmission of amoeba in the aquatic environment, but not on contact surfaces, was notable and significant. Further investigation and evaluation of transfer efficiency of amoeba from environment to different types of fresh produce and the virus titer recovered from samples are needed to better understand the effect and impact of *Acanthamoeba* on food safety issues related to fresh produce.

References

Barbeau, J., & Buhler, T. (2001). Biofilms augment the number of free-living amoebae in dental unit waterlines. *Res Microbiol, 152*(8), 753-760.

Barker, J., & Brown, M. R. (1994). Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. *Microbiology, 140 (Pt 6),* 1253-1259.

Beattie, T. K., Tomlinson, A., McFadyen, A. K., Seal, D. V., & Grimason, A. M. (2003). Enhanced attachment of *Acanthamoeba* to extended-wear silicone hydrogel contact lenses: a new risk factor for infection? *Ophthalmology, 110*(4), 765-771.

Bernard, H., Faber, M., Wilking, H., Haller, S., Höhle, M., Schielke, A., et al. (2014). Large multistate outbreak of norovirus gastroenteritis associated with frozen strawberries, Germany, 2012. *Euro Surveill, 19*(8), 20719.

Carrias, J. F. and T. Sime-Ngando (2009). Bacteria, Attached to Surfaces. Encyclopedia of Inland Waters. *Academic Press, Oxford* 182-192

Chappell, C. L., Wright, J. A., Coletta, M., & Newsome, A. L. (2001). Standardized method of measuring *Acanthamoeba* antibodies in sera from healthy human subjects. *Clin Diagn Lab Immunol, 8*(4), 724-730.

Chekabab, S. M., Daigle, F., Charette, S. J., Dozois, C. M., & Harel, J. (2012). Survival of enterohemorrhagic *Escherichia coli* in the presence of *Acanthamoeba castellanii* and its dependence on Pho regulon. *Microbiologyopen, 1*(4), 427-437.

Cirillo, J. D., Falkow, S., & Tompkins, L. S. (1994). Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infect Immun, 62*(8), 3254-3261.

Cordingley, J. S., Wills, R. A., & Villemez, C. L. (1996). Osmolarity is an independent trigger of *Acanthamoeba castellanii* differentiation. *J Cell Biochem, 61*(2), 167-171.

Cursons, R. T., Brown, T. J., Keys, E. A., Moriarty, K. M., & Till, D. (1980). Immunity to pathogenic free-living amoebae: role of humoral antibody. *Infect Immun, 29*(2), 401-407.

Danes, L., & Cerva, L. (1981). Survival of polioviruses and echoviruses in *Acanthamoeba castellanii* cultivated in vitro. *J Hyg Epidemiol Microbiol Immunol, 25*(2), 169-174.

DeWaal, C. S. & Bhuiya, F. (2007). Outbreaks by the numbers: fruits and vegetables 1990-2005. *Center of Science and the Public Interest 10*

Douesnard-Malo, F., & Daigle, F. (2011). Increased persistence of *Salmonella enterica* serovar Typhi in the presence of *Acanthamoeba castellanii*. *Appl Environ Microbiol, 77*(21), 7640-7646.

Ethelberg, S., Lisby, M., Bottiger, B., Schultz, A. C., Villif, A., Jensen, T., et al. (2010). Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010. *Euro Surveill, 15*(6).

Fankhauser, R. L., Noel, J. S., Monroe, S. S., Ando, T., & Glass, R. I. (1998). Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. *J Infect Dis, 178*(6), 1571-1578.

Gibson, K. E., Crandall, P. G., & Ricke, S. C. (2012). Removal and transfer of viruses on food contact surfaces by cleaning cloths. *Appl Environ Microbiol, 78*(9), 3037-3044.

Gibson, K. E., & Schwab, K. J. (2011). Tangential-flow ultrafiltration with integrated inhibition detection for recovery of surrogates and human pathogens from large-volume source water and finished drinking water. *Appl Environ Microbiol, 77*(1), 385-391.

Gourabathini, P., Brandl, M. T., Redding, K. S., Gunderson, J. H., & Berk, S. G. (2008). Interactions between food-borne pathogens and protozoa isolated from lettuce and spinach. *Appl Environ Microbiol, 74*(8), 2518-2525.

Hall, A. J., Eisenbart, V. G., Etingüe, A. L., Gould, L. H., Lopman, B. A., & Parashar, U. D. (2012). Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008. *Emerg Infect Dis, 18*(10), 1566-1573.

Huws, S. A., McBain, A. J., & Gilbert, P. (2005). Protozoan grazing and its impact upon population dynamics in biofilm communities. *J Appl Microbiol, 98*(1), 238-244.

Khan, N. A. (2003). Pathogenesis of *Acanthamoeba* infections. *Microb Pathog, 34*(6), 277-285.

Khan, N. A. (2006). *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiol Rev, 30*(4), 564-595.

Le Guyader, F. S., Mittelholzer, C., Haugarreau, L., Hedlund, K. O., Alsterlund, R., Pommepuy, M., et al. (2004). Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. *Int J Food Microbiol, 97*(2), 179-186.

Marciano-Cabral, F., & Cabral, G. (2003). *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev, 16*(2), 273-307.

Mattana, A., Serra, C., Mariotti, E., Delogu, G., Fiori, P. L., & Cappuccinelli, P. (2006). *Acanthamoeba castellanii* promotion of in vitro survival and transmission of coxsackie b3 viruses. *Eukaryot Cell, 5*(4), 665-671.

Pönkä, A., Maunula, L., von Bonsdorff, C. H., & Lyytikäinen, O. (1999). An outbreak of calicivirus associated with consumption of frozen raspberries. *Epidemiol Infect, 123*(3), 469-474.

Rude, R. A., Jackson, G. J., Bier, J. W., Sawyer, T. K., & Risty, N. G. (1984). Survey of fresh vegetables for nematodes, amoebae, and *Salmonella. J Assoc Off Anal Chem, 67*(3), 613-615.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., et al. (2011). Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis, 17*(1), 7-15.

Scheid, P., & Schwarzenberger, R. (2012). *Acanthamoeba* spp. as vehicle and reservoir of adenoviruses. *Parasitol Res, 111*(1), 479-485.

Schuster, F. L., & Visvesvara, G. S. (2004). Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int J Parasitol, 34*(9), 1001-1027.

Seal, D. V., Kirkness, C. M., Bennett, H. G., Peterson, M., & Group, K. S. (1999). *Acanthamoeba* keratitis in Scotland: risk factors for contact lens wearers. *Cont Lens Anterior Eye, 22*(2), 58-68.

Turner, N. A., Russell, A. D., Furr, J. R., & Lloyd, D. (2000). Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii*. *J Antimicrob Chemother, 46*(1), 27-34.

Visvesvara, G. S., Smith, P. D., Healy, G. R., & Brown, W. R. (1980). An immunofluorescence test to detect serum antibodies to *Giardia lamblia*. *Ann Intern Med, 93*(6), 802-805.

Weisman, R. A. (1976). Differentiation in *Acanthamoeba castellanii*. *Annu Rev Microbiol, 30,* 189-219.

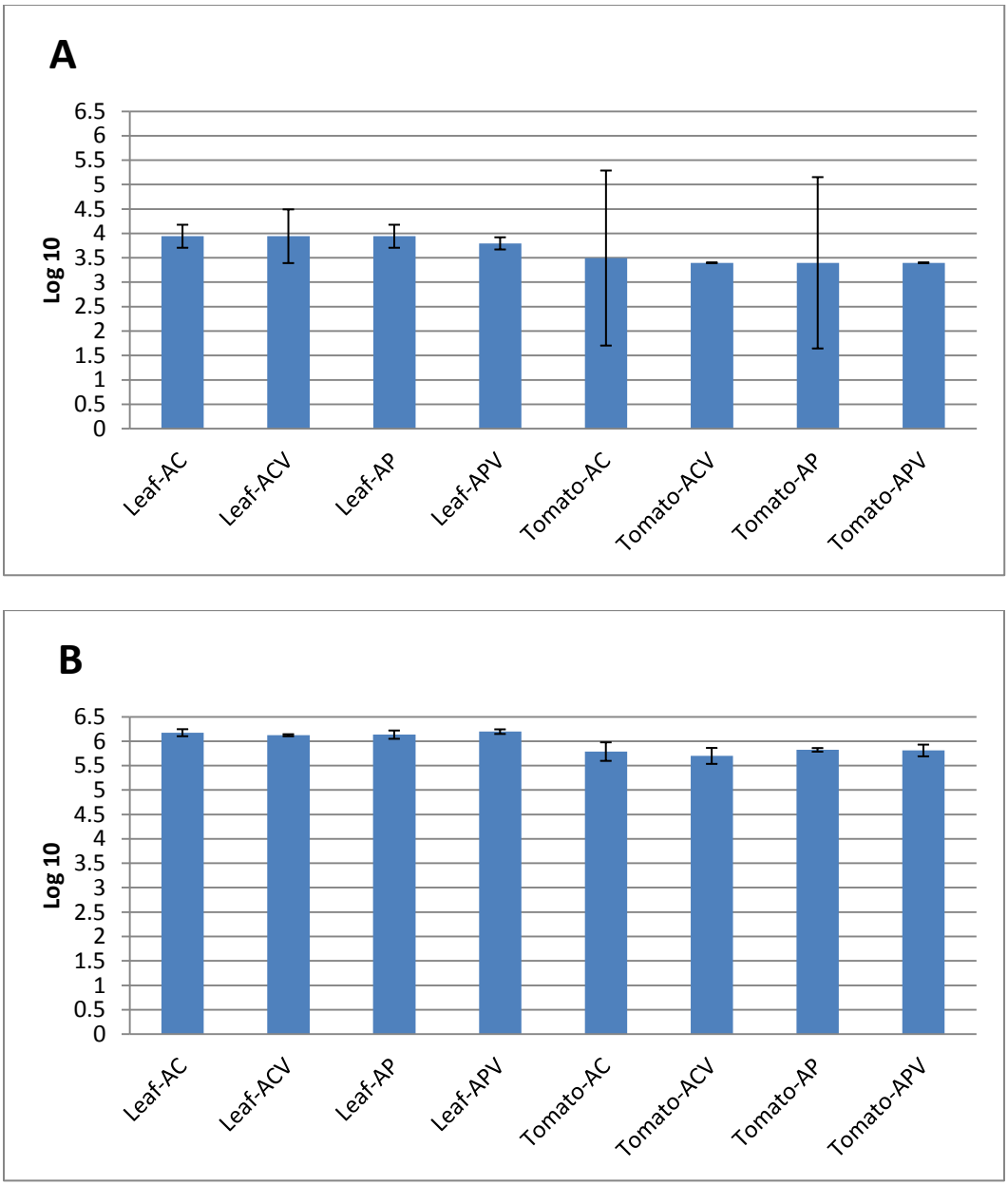

Figure 1. Number of amoeba eluted from fresh produce samples in water containing low (A) and high (B) concentration of *Acanthamoeba*. Leaf: baby kale and spinach; Tomato: cherry tomatoes; AC: *A. castellanii*; AP: *A. polyphaga*; ACV: virus-inoculated *A. castellanii*; APV: virus-inoculated *A. polyphaga*. Values are means ± standard errors from at least two experiments.

Chapter 4: Overall Conclusion

Based on environmental stability (i.e. persistence in water and on fomite surfaces), resistance to conventional cleaning agents (e.g., chlorine bleach, quaternary ammonium compounds) (Keswick et al., 1985; Duizer et al., 2004), and low infectious dose (18-1,000 virus particles) (Teunis et al., 2008), human noroviruses (HuNoV) are responsible for more than 95% of viral gastroenteritis outbreaks and over 50% of all gastroenteritis outbreaks worldwide (Karst, 2010). In addition, HuNoV are the most common cause of foodborne disease outbreaks in the United States. The most common food vehicles implicated in HuNoV outbreaks include primarily ready to eat (RTE) foods and foods with minimal processing including shellfish, fresh produce such as leafy greens, and deli sandwiches. Within these food vehicles, fresh produce accounts for 64% of foodborne norovirus outbreaks (DeWaal and Bhuiya, 2007). Even though the food vehicles can frequently be identified during a norovirus outbreak investigation, the route of transmission and point of contamination are often unknown if a food handler is not implicated. Very little attention has been paid to environmental transmission of HuNoV with respect to the food supply chain, specifically for fresh produce production; however, there is evidence that outbreaks can occur due to contamination at the point of production and harvest (Le Guyader et al., 2004; Ethelberg et al., 2010; Sarvikivi et al., 2012) as opposed to the point of preparation (i.e. food handler).

One potential pathway for HuNoV to contaminate fresh produce is through interactions with other microorganisms present in the soil and water environments or even through simple

particle association (Sobsey and Meschke, 2003). In the research presented here, we hypothesized that HuNoV can associate with free-living amoeba (FLA) and then possibly be transmitted to fresh produce through contaminated soil and water sources. Based on previous reports of bacterial and viral pathogen relationships with FLA (Khan, 2006; Thomas et al., 2010), *Acanthamoeba* spp. was selected for this research on FLA and HuNoV interactions. *Acanthamoeba* is a genus of common FLA and is ubiquitous in air, soil, and water environments. *Acanthamoeba* can also be found in chlorinated swimming pools, drinking water, cooling tower, natural thermal water, hospital water network and even marine water. In addition, FLA have been isolated from fresh produce such as leafy vegetables, tomatoes and fruits (Rude et al., 1984; Gourabathini et al., 2008). Therefore, this master's thesis focused on understanding a piece of the puzzle with respect to HuNoV transmission pathways in the environment.

The first manuscript (Chapter 2) focused on the investigation of interactions between *Acanthamoeba* and the HuNoV surrogate, murine norovrus type 1 (MNV-1). This investigation included evaluation and characterization of virus-amoeba interaction. For the initial evaluation, we analyzed virus titer associated with amoeba after virus-amoeba incubation and observed the effect of virus incubation on amoeba growth. To further characterize the interaction(s), we analyzed the change of virus titer associated with amoeba over time and through a complete amoeba life cycle (trophozoite → cyst → trophozoite). Last, we observed the location of associated virus in/on amoeba using a double immunofluorescence assay. Overall, these data demonstrated that MNV-1 can associate with *A. castellanii* for at least 8 days and maintain infectivity through a complete life cycle (i.e. encystment and excystment) without affecting the morphology and normal growth of amoeba. Moreover, the immunofluorescence stain indicated that MNV-1 can translocate from the amoeba surface and internalize in the amoeba after 24

hours of amoeba-virus incubation. Since *Acanthamoeba* is resistant to many harmful environmental factors especially in the cyst form (Weisman, 1976; Cordingley et al., 1996; Turner et al., 2000), these interactions might be favorable for virus survival. This is the first study to report on the interaction of *Acanthamoeba* with a MNV-1—a surrogate for the study of HuNoV—and to determine the localization of the interaction in the amoeba over a 24-hour period. This increase in knowledge related to norovirus interactions with environmental microorganisms will lead to more in depth studies related to the transmission of HuNoV in the environment.

Establishing the interaction of MNV-1 with amoeba set the stage for the second portion of this master's thesis. The second manuscript (Chapter 3) focused on evaluating amoeba transfer from the environment (water and fomite surfaces) to fresh produce. This evaluation included observing the transfer of both amoeba and amoeba with associated virus from water and food contact surfaces (i.e. stainless steel and PVC conveyor material) containing different concentrations of amoeba to fresh produce sample including baby leaf salad and cherry tomato. This investigation showed that though the transfer of amoeba from contact surfaces to fresh produce was very limited, the transfer of amoeba in an aquatic environment was notable. In addition, although amoeba transfer efficiency might depend on sample surface area and surface texture, the data showed that virus association did not affect the transfer efficiency.

In this study, we demonstrated that 1) *A. castellanii* trophozoites had the ability to associate with MNV-1 and carry those viruses through their life cycle 2) MNV-1 could move from amoeba surface to inside amoeba after virus association with *A. castellanii* and at the same time, remain infectious and 3) the transfer of virus-associated *A. castellanii* from the aquatic environment to fresh produce samples was notable. Based on these data, it is possible that *A.*

castellanii has the potential to carry HuNoV that may be present in aqueous environments and even transfer to fresh produce during irrigation, washing, or post-harvest processing.

References

Carrias, J. F. and T. Sime-Ngando (2009). Bacteria, Attached to Surfaces. Encyclopedia of Inland Waters. *Academic Press, Oxford* 182-192

Cordingley, J. S., Wills, R. A., & Villemez, C. L. (1996). Osmolarity is an independent trigger of *Acanthamoeba castellanii* differentiation. *J Cell Biochem, 61*(2), 167-171.

DeWaal, C. S. & Bhuiya, F. (2007). Outbreaks by the numbers: fruits and vegetables 1990-2005. *Center of Science and the Public Interest 10*

Duizer, E., Bijkerk, P., Rockx, B., De Groot, A., Twisk, F., & Koopmans, M. (2004). Inactivation of caliciviruses. *Appl Environ Microbiol, 70*(8), 4538-4543.

Gourabathini, P., Brandl, M. T., Redding, K. S., Gunderson, J. H., & Berk, S. G. (2008). Interactions between food-borne pathogens and protozoa isolated from lettuce and spinach. *Appl Environ Microbiol, 74*(8), 2518-2525.

Karst, S. M. (2010). Pathogenesis of noroviruses, emerging RNA viruses. *Viruses, 2*(3), 748-781.

Keswick, B. H., Satterwhite, T. K., Johnson, P. C., DuPont, H. L., Secor, S. L., Bitsura, J. A., et al. (1985). Inactivation of Norwalk virus in drinking water by chlorine. *Appl Environ Microbiol, 50*(2), 261-264.

Khan, N. A. (2006). Acanthamoeba: biology and increasing importance in human health. *FEMS Microbiol Rev, 30*(4), 564-595.

Le Guyader, F. S., Mittelholzer, C., Haugarreau, L., Hedlund, K. O., Alsterlund, R., Pommepuy, M., et al. (2004). Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. *Int J Food Microbiol, 97*(2), 179-186.

Rude, R. A., Jackson, G. J., Bier, J. W., Sawyer, T. K., & Risty, N. G. (1984). Survey of fresh vegetables for nematodes, amoebae, and *Salmonella*. *J Assoc Off Anal Chem, 67*(3), 613-615.

Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., et al. (2012). Multiple norovirus outbreaks linked to imported frozen raspberries. *Epidemiol Infect, 140*(2), 260-267.

Teunis, P. F., Moe, C. L., Liu, P., Miller, S. E., Lindesmith, L., Baric, R. S., et al. (2008). Norwalk virus: how infectious is it? *J Med Virol, 80*(8), 1468-1476.

Thomas, V., McDonnell, G., Denyer, S. P., & Maillard, J. Y. (2010). Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. *FEMS Microbiol Rev, 34*(3), 231-259.

Turner, N. A., Russell, A. D., Furr, J. R., & Lloyd, D. (2000). Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii*. *J Antimicrob Chemother, 46*(1), 27-34.

Weisman, R. A. (1976). Differentiation in *Acanthamoeba castellanii*. *Annu Rev Microbiol, 30*, 189-219.

Appendix: Institutional Biosafety Committee Protocol Approval

UNIVERSITY OF ARKANSAS

Office of Research Compliance

January 11, 2013

MEMORANDUM

TO: Dr. Kristen Gibson

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 13017

Protocol Title: "Understanding Environmental Reservoirs and Prevalence of Norovirus Surrogates to Reduce Impact on Public Health"

Approved Project Period: Start Date: January 10, 2013
Expiration Date: January 09, 2016

The Institutional Biosafety Committee (IBC) has approved Protocol 13017, "Understanding Environmental Reservoirs and Prevalence of Norovirus Surrogates to Reduce Impact on Public Health". You may begin your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199