The Prevalence and Potential Role of Human Cytomegalovirus, Epstein Barr Virus and Mouse Mammary Tumor Virus in Pakistani Acute Appendicitis Patients

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2017

The Prevalence and Potential Role of Human Cytomegalovirus, Epstein Barr Virus and Mouse Mammary Tumor Virus in Pakistani Acute Appendicitis Patients

A thesis submitted in partial fulfillment of the requirement for the

Degree of Masters of Philosophy

In

Applied Biosciences

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2017

FORM TH-4

National University of Sciences & Technology

MASTER THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by: Sara Hassan regn no: 00000117753.

Titled: 'Viral etiology of Acute Appendicitis' be accepted in partial fulfillment of the requirements for the award of (Grade A) Masters MS degree.

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ACKNOWLEDGEMENTS

All praise to Allah Almighty who bequeathed me with the courage, strength and perseverance throughout the course of this research work.

I take great privilege in expressing my gratitude to my supervisor **Dr. Hajra Sadia**, who mentored me throughout my MS and primarily during the course of research. I am truly grateful for her priceless guidance, admirable work ethic and relentless support, in facilitating us with the opportunity, resources, and the constructive environment to perform this research.

I would like to express my gratitude to **Dr. Peter John** Principal, ASAB NUST Dr. **Attya Bhatti**, for their concern and invaluable guidance throughout my MS.

I would like to acknowledge **Dr. Jahangir Sarwar,** Head of Surgery at Holy Family Hospital Rawalpindi for facilitating us with diagnosis and sampling. I appreciate all the patients who volunteered to participate in my research by giving the consent for blood samples.

My labmates **Umm-e Aimen**, **Faiza Saleem**, **Behroz Naeem**, my brothers in arms for their help, motivation and endurance in troublesome times. My seniors Wasifa Naushad, Orooj Surraiya and Rubina Daad for their support and encouragement.

In the end, I would like to express my gratitude to my mother **Saima Athar**-to whom Im eternally indebted, my muse, everything I do, my victories and accomplishments are all for you. Thank you for being my confidant, my best friend, my enabler and my shield. I am grateful to my father **Hussain Athar** for his unwavering faith, relentless support and for instilling the sense of entitlement in me and making me set higher goals for myself.

SARA HASSAN

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LIST OF ACRONYMS

%	**Percentage**
~	**Approximately**
°C	**Degree Celsius**
Bp	**Base Pair**
CMV	**Cytomegalovirus**
EBV	**Epstein Barr virus**
EDTA	**Ethylene Diamine Tetra Acetic Acid**
PCR	**Polymerase Chain Reaction**
Kb	**Kilo Base**
MgCl2	**Magnesium Chloride**
mL	**milli Liter**
mM	**milli Molar**
TAE	**Tris Actate EDTA**
Taq	**Thermus aquaticus**
UV	**Ultraviolet**
TLR	**Toll like receptor**

ABSTRACT

Acute appendicitis is one of the most common cause of abdominal surgeries. It is responsible for approximately 72,000 deaths globally per year. Yet its etiology has still not been elucidated. The study was conducted to verify if the viruses are the etiological agents of acute appendicitis. For this purpose, the incidence of cytomegalovirus (CMV), Epstein-Barr virus (EBV), mouse mammary tumor virus (MMTV) was studied in Pakistani acute appendicitis patients. Genomic DNA was extracted from the peripheral blood samples of thirty patients suffering from acute appendicitis who had undergone appendectomies. Qualitative Polymerase chain reaction (PCR) assays were employed to screen DNA samples of acute appendicitis patients for EBV, CMV and MMTV. Thirty age and gender matched peripheral blood samples that had no clinical manifestations related to viral infections were taken as controls. CMV was the most frequently detected virus 13.3% , followed by EBV 10% and least frequently detected was MMTV 3.3%. This indicates that these viruses maybe the etiological agents of acute appendicitis and maybe associated with the pathogenesis of acute appendicitis. To further evaluate and confirm these findings additional studies need to be conducted.

CHAPTER 1

INTRODUCTION

Appendicitis is a common clinical condition defined as the inflammation of the appendix (Graffer *et al*., 1996). The appendix - a part of the gastrointestinal tract, is a 3 1/2-inch blind tube of tissue that extends from the large intestine. The role of appendix in humans has been elusive and undefined and its removal does not have any bad consequences. However, the inflammation of the appendix in humans has severe consequences and results in death (Hobler *et al.,* 1996). In spite of the present diagnostic and therapeutic advancements, appendicitis remains a clinical emergency and is one of the more common causes of acute abdominal pain, treated only by open or closed appendectomy by laparoscopy.

About 250,000 cases of acute appendicitis are reported in The United States and 40,000 in the United Kingdom annually. Acute appendicitis causes considerable mortality and morbidity. In 2013 about 16 million cases of appendicitis occurred. This resulted in 72,000 deaths globally (Weir *et al.,* 2013).

1.1 Symptoms and manifestations:

Acute Appendicitis is manifested by nausea, vomiting, loss of appetite anorexia, loss of weight as a consequence of loss of appetite, abdominal pain, swelling of the abdomen, constipation, diarrhea, flatulence, severe abdominal cramps and fever. To ensure correct diagnosis X-rays, CT scans and ultrasounds are performed to diagnose appendicitis. Pregnancy tests are done to rule out pregnancy (Graffeo *et al.,* 1996).

1.2 Etiological agents:

In the multifactorial etiology of acute appendicitis, the most common cause is the obstruction of the appendix by appendicolith. Fecolith is a stone made by the calcification of faeces present in the appendix. Secondly, the formation of tumors inside the appendix also obstructs the appendix leading to acute appendicitis. Bacterial infections caused by *E. coli*, Yersinia species; Shigella, Salmonella and Parasitic infestations caused by Entamoeba histolytica have also been known to cause acute appendicitis. Inflamed lymphoid tissue from a viral infection, parasites, gallstone, may also cause the blockage (Jones et al., 1985, Norman *et al.,* 2000). Various studies have implicated enteric and systemic viruses like measles virus, adenovirus and cytomegalovirus (CMV) and Epstein Barr Virus (EBV) in the pathogenesis of acute appendicitis as these viruses cause a reaction of the lymphoid follicle. Cytomegalovirus appendicitis following acute Epstein-Barr virus infection was reported in an immunocompetent patient. A rare case of post-renal transplant large T-cell lymphoma was reported to have an unusual presentation of acute appendicitis along with Epstein Barr Virus-positivity. Cytomegalovirus appendicitis following acute Epstein-Barr virus infection was reported in an immunocompetent patient.

1.2.1 Acute Appendicitis And Mouse Mammary Tumor Viruses

Mouse mammary Tumor virus is a retrovirus discovered in 1939 by Bittner. MMTV is a replication competent B type virus which causes mammary carcinomas. MMTV is responsible for the vertical transmission of breast cancer by nursing. MMTV has the potential to infect and integrate in the cell lines of human epithelial breast cancer. MMTV is carried by several mice strains endogenously however, but it is also transmitted through milk from mother to newborn pups (vertical transmission). Viruses are then transmitted endogenously via germ line. The ingestion of MMTV into the intestinal tract is followed by invasion through dendritic cells and lymphocytes into the Peyer's patch. These

lymphocytes move to the spleen and stay dormant for long time periods and subsequently move to the mammary glands randomly integrating into DNA of host's mammary epithelial cells. This random integration of MMTV provirus occurs near Wnt and fgf genes as a consequence of which inappropriate oncogenic expression and uncontrolled proliferation of mammary cells occurs leading to hyperplasia. There is a possibility of a zoonotic transmission of MMTV to humans as there is a worldwide distribution of MMTV sequences seen in human populations with breast cancer.

1.2.2 Acute Appendicitis and Cytomegalovirus

Human Cytomegalovirus is the most complex beta- herpes virus also known as Human Herpes Virus 5. It is a highly prevalent virus in human population ranging from 30% to 90% seroprevalence (Tania *et al.,* 2009, Jessica *et al.,* 2010). It is transmitted through vertical via by placenta to developing fetus and from milk to newborns. Horizontal means it is transmitted through body fluids, transplants and sexual contact. The resulting infections in healthy individuals are asymptomatic. However, to immunocompromised individuals CMV infections are fatal. CMV is implicated in congenital disorders due to primary or reactivated maternal infections during pregnancy which consequently lead to mental retardation and neurodevelopment disorders in the children.

1.2.3 Acute Appendicitis and Epstein Barr Virus

Epstein Barr Virus is also designated as human herpes virus 4 (HHV-4) belongs to human herpes virus family. It is one of the most prevalent viruses in humans. EBV is implicated in the pathogenesis of infectious mononucleosis also known as glandular fever. Moreover, it is also implicated as an etiological agent of cancer for instance Hodgkin's lymphoma and gastric cancers. EBV infections are mostly asymptomatic with occasional manifestations like fever, swollen lymph nodes, enlarged spleen and sore throat.

1.3 Pathophysiology of Acute Appendicitis:

Acute appendicitis is primarily caused due to the obstruction of the hollow region of the appendix. Following its obstruction, the appendix is filled with mucous and consequently swells up. The continued mucus secretion in the appendix leads to an increase in the pressure of on the appendix walls as well as within the lumen. This increase in pressure leads to thrombosis as well as occlusion in the smaller blood vessels eventually leading to the stasis of lymphatic flow. With the progression of the occlusion in the appendix blood vessels the appendix first turns ischemic and finally it becomes necrotic. With the leakage of the bacteria through the necrotic walls of the appendix, pus forms begin around and inside the appendix this process is called suppuration. All these events lead to the appendiceal rupture or bursting causing peritonitis. Peritonitis may cause sepsis which ultimately leads to death. These conditions manifest themselves in symptoms like abdominal pain and other associated symptoms (Schwartz *et al.*, 2010).

1.4 Treatments of Acute Appendicitis:

In spite of the present diagnostic and therapeutic advancements the treatments prescribed for appendicitis are antibiotics. However, appendicitis remains a clinical emergency and in order to avert a perforated appendix the more common causes of acute abdominal pain, treated only by appendectomy (Schwartz *et al.*, 2010).

Acute Appendicitis is reported to have a high incidence in Pakistan. The ultimate treatment that healthcare professionals resort to is open or laparoscopic appendectomy. This study has the potential to elucidate that whether viruses like CMV, EBV are etiological agents and contribute to the pathogenesis of acute appendicitis. If the role of viruses in causing acute appendicitis is confirmed, the novel viral based diagnostic tests will be designed for acute appendicitis. Moreover, acute appendicitis will be cured by antiviral therapy and there will be no need for a surgical appendectomy.

This study will help understand the pathogenesis and etiology of acute appendicitis. Previously, very little research has been done to speculate and confirm the role of viruses in the etiology and pathogenesis of acute appendicitis. If the role of viruses is confirmed this will lead to a new diagnostic test for testing viruses responsible for causing acute appendicitis. In the cases where the acute appendicitis is caused by viruses' antiviral therapy instead of an appendectomy could be used as treatments.

OBJECTIVE

- To investigate whether viruses are the etiological agents of acute appendicitis DNA samples of acute appendicitis were screened for EBV, CMV and MMTV sequences.

CHAPTER 2

REVIEW OF LITERATURE

Acute appendicitis is the inflammation of the appendix. It is notorious for being the most common reasons for abdominal surgeries performed on emergency basis. In 2013 about 16 million cases of appendicitis occurred. This resulted in 72,000 deaths globally. About 250,000 reports appendicitis are reported in The United States each year and the mortality, morbidity, and economic cost of appendectomy cannot be ignored (Weir *et al.*,2013).

In spite of the present diagnostic and therapeutic advancements, appendicitis remains a clinical emergency and is one of the more common causes of acute abdominal pain, treated only by open or closed appendectomy by laparoscopy. It primarily affects adolescents and young adults during the second and third decades of their lives (Alder *et al.*,2000) . Acute appendicitis has a male to female ratio 1.3:1. It is more prevalent in males compared to females. In children subjected to appendectomies 77% range from 6 to 18 years in age (Richardsen *et al.,* 2015).

The apparent causes of appendicitis are the luminal obstruction created in the appendiceal lymphoid follicle. The obstruction can be caused by megaliths, foreign bodies, and cancerous tumors. Moreover, bacterial infections for instance *Escherichia coli,* Shigella, Yesinia or Salmonella can cause Acute Appendicitis. Similarly, parasitic infestations for instance *Entamoeba histolytica* or systemic viral diseases like cytomegalovirus CMV and adenovirus can lead to inflammation of the appendix (Jones *et al.,* 1992).

Acute Appendicitis can be diagnosed by tests like White blood cell count. C-reactive protein concentrations of the blood serum can also help diagnose acute appendicitis. Moreover, newly discovered inflammatory markers nuclear factor (NF)-kappaB, interleukin lL-6 are also being studied as potential markers for acute appendicitis detection (Filiz *et al.,* 2010).

Herpes viruses- a highly diverse family of DNA viruses, all of them have the ability to illicit latent infections throughout the lives of their hosts. During childhood, they cause primary infections in their hosts. However, their role in acute appendicitis has not been elucidated as yet. It has been reported that CMV infection is associated with acute appendicitis in patients suffering from AIDS. Moreover, in an immunocompetent individual acute appendicitis occurred after an EBV infection (Zakafani *et al.,* 2004).

In another case CMV was reported to in an acute appendicitis patient who was non-immunosuppressed and non-HIV negative. In another study the molecular analysis of and peripheral blood lead to the discovery of CMV and EBV DNA in patients of acute appendicitis suggesting that viral infection may be implicated in the pathogenesis of acute appendicitis (Katzoli *et al.,* 2009).

2.1 Acute Appendicitis and Human Cytomegalovirus Virus

2.1.1. Pathology and Viral Features of CMV

Human cytomegalovirus also known as Human Herpes Virus HHV5 is one of the most complex viruses of beta herpersviridae-a herpes virus subfamily. HCMV has a high prevalence in human population infecting more than 40% of the world's population. Depending on socioeconomic, geographic and ethnic backgrounds its seroprevalence ranges from 30-90% (Tania *et al.,* 2009, Jessica *et al.,* 2010).

CMV holds the potential for both vertical and horizontal transmission. It is transmitted horizontally via bone and organ transplants, blood transfusions, saliva and sexual contact. Vertical transmission is via breast milk or transplacental transmission to the fetus. CMV infects dendritic cells, hepatocytes, fibroblasts, endothelial cells, smooth muscle cells and epithelial cells of the host cells. This broad cell tropism enables systemic spread of the virus in the host body (Sinzer *et al.*, 2008).

HCMV infections do not manifest them as they're mostly asymptomatic and subsequently lead to a lifelong latency in the host. Moreover, they are a cause of considerable morbidity and mortality primarily due to their infections in immunocompromised individuals like AIDS patients or immunosuppressed individuals like recipients of organ transplants (Massimillano *et al.*, 2011).

In the United States 0.2-2% children suffer from congenital HCMV infection annually. Congenital HCMV infections, due to maternal infection or reactivation, subsequently lead to neurodegenerative disease, mental retardation and hearing loss. HCMV is implicated in causing the disease either by direct chromosomal injury or by altering the gene expression (Maxim *et al.*, 2009).

2.1.2 Structure and genome of HCMV

CMV is a double stranded DNA virus consisting of one the largest genomes of approx. 240kb with around 200 open reading frames. Its possesses the genomic organization characteristic of a herpes viruses, consisting of unique long regions (UL) and unique short regions (US), flanked by two sets of inverted repeats (Massimillano *et al.*, 2011).

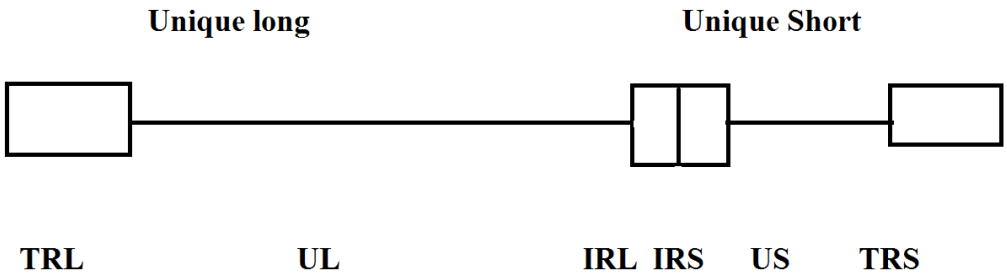

Figure 2.1 Schematic Representation of HCMV genome

HCMV has a 125 nm icosahedra capsid which encapsulates its 240kb double stranded DNA. The capsid is surrounded by a protein rich tegument composed of mRNA viral proteins and certain host proteins. The tegument is in turn encapsulated by a lipid bi layered envelop which is host derived. This envelop is based on 20 glycoproteins that are encoded by the virus. The glycoproteins of the envelop are significant in the attachment and internalization of the virus (Shang *et al.,* 2000).

2.1.3. Mechanism of Infection

Of all the glycoproteins Glycoprotein B is the most significance in the host's innate and adaptive immunity. Various studies have reported that the interaction of gpB with cell surface receptors like Toll Like Receptor 2 (TLR2) and Platelet Derived Growth Factor alpha (PDGF) are responsible for altering transcription of various cellular genes (Liliana et al.,2006). Binding of gpB to TLR2 plays and important response in orchestrating the innate immune response of the host, against HCMV through the stimulation of proinflammatory cytokine (Karl *et al*, 2006).

HCMV binding to TLR2 initiates the TLR2 signalling cascade by stimulating the recruitment of adapter protein MYD88. As a consequence of which the serine/threonine kinase IL1 receptor associated kinases (IRAK 1 and 2) are phosphorylated and activated. IRAK 1 dissociates form the complex after phosphoylation and subsequently associates

with TRAF-6. TRAF-6 is responsible for activating TAK-1 and phosphorylates IKK which is complexes with NF-kB (Wang *et al.,* 2001). After phosphorylation of IKK it is dissociated form NF-kB. NFkB is activated and translocates into the nucleus and transcribes genes. (Joelle *et al*, 2005).

2.1.4. CMV involvement in Acute Appendicitis

Cytomegalovirus is a systemic virus and has the potential of affecting entire organ systems. Cytomegalovirus has been implicated in ulcerative colitis and appendicitis (Sylvie *et al.,* 2013). CMV and EBV were the most frequently reported viruses in a study conducted to detect herpes viruses in children suffering from acute appendicitis (Katzoli *et al.*, 2009). It was reported that acute appendicitis developed prior to a Cytomegalovirus and EBV infection in an immunocompetent patient (Zakanafani *et al.*, 2004).

2.2. Acute Appendicitis and Epstein Barr Virus

2.2.1. Pathology and Viral Features of EBV

Epstein Barr virus also designated as (HHV-4) human herpesvirus 4 comes under the subfamily of herpes virus - lymphocryptovirus. This ubiquitous virus infects about 90% of the population (Deshpande *et al*., 2002).

EBV holds the potential for both vertical and horizontal transmission. It is transmitted horizontally through body exchange of body fluids especially saliva rendering the oral mucosa the targets of early life primary infections (Glaser *et al.,* 2004).

EBV infections do not manifest them as they're mostly asymptomatic. Prior to the infection lifelong latency persists in the host B cells. EBV is implicated in the pathogenesis of infectious mononucleosis, cancers Hodgkin's disease and gastric cancer. It causes considerable morbidity and mortality in immunocompromised individuals causing AIDS associated lymphomas patients and lymphoproliferative disorders in recipients of organ

transplants. Moreover, autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus, and multiple sclerosis are associated with EBV infection (Deshpande *et al.*, 2002).

2.2.2. Structure and Genome of EBV

Epstein Barr Virus is a double stranded DNA virus. It is 172 kb approximately. The EBV genome comprises of 0.5kb of long and short unique sequences of internal repeats sequences and terminal direct repeats. The genome encodes for approximately 100 genes (Young *et al.*, 2003). The diameter of EBV ranges from 122-180 nm, with an octahedral capsid enclosed in a tegument layer. The tegument is in turn encapsulated by an envelope which is host derived. This envelop is based on glycoproteins that are encoded by the virus. Glycoproteins of the envelop are significant in the attachment and internalization of the virus (Eligio *et al.*, 2010).

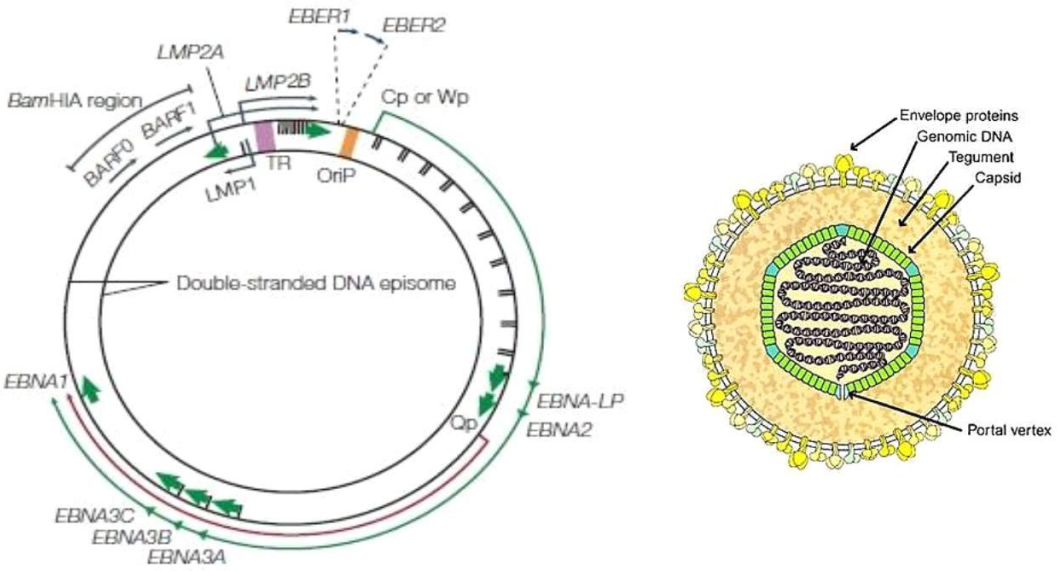

Figure2.2: Epstein Barr Virus structure and genome. (Young and Murray, 2003)

2.2.3. Mechanism of Infection of EBV

EBV mainly targets epithelial cells, B cells and T cells. In the case of B cells glycoprotein 350 of EBV binds to host CD21. Subsequently, MHC II, with the assistance of gH, gp42 and gL fuses both the membranes. In the case of epithelial cells, BMRF2 of EBV bind to and beta1-integrins of the host cell to initiate internalization. Subsequently, MHC II, with the assistance of gH, gp42 and gL fuses both the membranes (Draborg *et al.*, 2013).

Epstein Barr Virus life cycle is divided into two phase's i.e. latent and lytic phase. Latent phase is characterized by the residence of EBV in the host cells without producing virions. The virus contains a set of specialized genes to select the suitable mode of living depending upon various external and internal factors, such as stress and nutrient availability etc. EBV has a complex mechanism which allows it to switch among different patterns of latency, a phenomenon known as latency types (Eligio *et al.,* 2010). EBV Infection in both B lymphocytes and in epithelial cells are mostly latent in nature and subsequently leads to lympho-proliferation. Latency of EBV latency leads to oncogenesis and immune evasion. EBV genome exists in the host cell as an extrachromosomal plasmid and hence escapes cytotoxic T cells (CTL) immune surveillance through down regulating certain genes that are target of CTLs (Deshpande *et al.,* 2002).

2.2.4. EBV involvement in Acute Appendicitis

CMV and EBV were the most frequently reported viruses in a study conducted to detect herpes viruses in children suffering from acute appendicitis (Katzoli *et al.,* 2009). It was reported that acute appendicitis developed prior to a Cytomegalovirus and EBV infection in an immunocompetent patient (Kanafani *et al.,* 2004). Acute Appendicitis was reported in an EBV infected individual suffering from mono nucleosis (Antonio *et al.,* 1990) CMV and EBV infections were reported in patient inflicted with acute appendicitis and B Cell Lymphoma (Ting-Ying *et al.,* 2010) In various studies conducted it has been reported that

Epstein-Barr Virus Infection is commonly reported in Inflamed gastrointestinal Mucosa (Julie *et al.,* 2013).

2.3. Acute Appendicitis and Mouse mammary tumor virus

2.3.1. Pathology and Viral features

Mouse mammary tumor virus is a replication competent virus belonging to the family Retroviridae and the genus Betaretrovirus. MMTV is vertically transferred from mice to pups via breast milk can be transferred either through an exogenous or endogenous route. Moreover, in case of endogenous infection the pups inheriting MMTV from their mother have proviral DNA inserted in the chromosomes of all their cells (Lawson *et al.,* 2010).

MMTV infects the human breast epithelial cell lines, integrates in the DNA and multiplies. MMTV is a well-documented etiological agent of breast cancer and tumors in field mice as well as experimental mice. Moreover, the presences of sequences of similarity with MMTV have been documented in 40% of American women inflicted with breast cancer (Wang *et al.,* 1995).

2.3.2. Structure and genome of MMTV

MMTV genome comprises of a single stranded RNA molecule. It was fully sequenced in the year 1998. The size of the MMTV genome is 8 kb and 7 genes are encoded by it. The MMTV genome is flanked by short direct repeats(R) on both sides. An MMTV virion contains a genomic RNA dimer and it is a B-type particle. The MMTV virion is encapsulated by helical nucleoproteins. The RNA dimer is surrounded by an icosahedral capsid for. The capsid is further encapsulated by an envelope composed by a transmembrane proteins and surface proteins (Kingsley *et al.,* 2008).

2.3.3. Mechanism of Infection of MMTV

MMTV may be present in mice endogenously. MMTV is passed on to the next generation by germ line transmission. Moreover, consuming milk containing lymphocytes carrying MMTV provirus leads to the entrance of virus into the gastro intestinal tract of the mice pups after which the virus infiltrates the lymphatic system and subsequently enters the payers patch where they infect the hosts lymphocytes and macrophages. These infected lymphocytes infiltrate the spleen and become inactive. These lymphoycytes leave the spleen and infect the host's mammary epithelial cells and cause mammary tumors (Callahan *et al.,* 2000).

In mice MMTV binds to transferring receptor 1(TfR1) which is an extracellular transmembrane protein as a consequence of which the virus receptor complex is endocytosed. However, the receptor with functional homology to TfR1 has not been discovered in humans but the ability of MMTV to infect a wide range of cells (Ross *et al.,* 2002).

The zoonotic transmission of MMTV into humans is because of the worldwide distribution of MMTV like gene sequences found in MMTV carrying Mus *domesticus*. Transmission of MMTV to humans is proposed to be due to the consumption of food infected by food and cereal contaminated by mice droppings (Dendy *et al.,* 2001).

2.3.4. MMTV involvement in Acute Appendicitis

A study conducted by Fernandez et al showed that the MCF-7 cell line containing the MMTV envelope showed an increased expression TGF and TNF genes that are responsible for causing inflammation in cells in comparison to the other subtype which did not contain MCF-7 subtype (Fernandez *et al.,*2006).

CHAPTER 3

MATERIAL AND METHODS

3.1 STUDY SUBJECTS

Clinically diagnosed patients of Acute Appendicitis were identified at the The Holy Family Hospital in Rawalpindi. These individuals underwent detailed clinical evaluation. Clinically diagnosed individuals suffering from Acute Appendicitis were interviewed for their clinical symptoms and manifestations and family history. Each participant was asked to fill a letter of consent (Annexure I) prior to participation. Information regarding age, ethnic background, and relevant health information was recorded in the designed questionnaire (Annexure II). Blood samples were collected from 30 patients of Acute Appendicitis. Blood samples of 30 healthy age and gender matched controls were also collected.

Inclusion Criteria of Samples: 30 Clinically Diagnosed patients of Acute Appendicitis were included in this study.

Inclusion Criteria of Controls: Samples of 30 healthy age and gender matched individuals were collected as controls for the study.

Clinical data of patients was recorded and reviewed before collecting the sample (Annexure B). The inclusion criteria of individuals participating in the study were individuals diagnosed with acute appendicitis. Patients with other diseases were excluded from the study. Blood samples were collected in sterile vacutainers with EDTA added as

anticoagulant. Age and gender matched control samples were also collected from healthy individuals who were not suffering from acute appendicitis

3.2 Genomic DNA extraction

DNA extraction from blood was performed following the Phenol chloroform method.

3.2.1 DNA extraction from venous blood

Genomic DNA extraction using blood samples, 750μL blood was taken in labelled, autoclaved, 1.5ml micro centrifuge tube. 750 μL of Solution A (lysis buffer composed of 0.32M sucrose, 1M Tris-HCl, Triton X100, 1M $MgCl_2$) was added to the micro centrifuge tube. The tube was subsequently inverted 3-4 times to ensure homogenization and kept at room temperature for 10 minutes followed by centrifugation for 1 minute at 14000rpm and the supernatant was decanted. nuclear pellet is subsequently suspended into 400 μL of the solution A 1 minute for 14000rpm and the supernatant was decanted. The resulting pellet is suspended into 12 μL of 20%SDS, 5μL proteinase K, 400 μL of Solution B (composition: 100mM NaCl at 7.4 pH, 4mM Na_2EDTA, 20mM Tris-HCl) and subjected to overnight incubation at 37°C.

Equal volumes of Phenol (solution C) and Solution D (composition: chloroform 24vol: isoamylalcohol vol1) were prepared and 500μL of this solution was added per tube and subjected to centrifugation at 13000rpm for 10 minutes. 500 μL of the resulting aqueous phase was 13000rpm for 10 minutes.

Aqueous layer was again transferred to a fresh tube and subjected to DNA precipitation by addition of 55 μL of 3M sodium acetate and 1mL ethanol. The tube was inverted 3-4 times and subjected to centrifugation at 13000rpm for 10 minutes. The supernatant was decanted off. The DNA pellet visible at the base of the tube is subjected to washing with 200 μL of 70% ethanol and centrifuged at 13000rpm for 7 minutes. The supernatant is decanted and pellet is dried on absorbent paper at 37°C for 30 minutes and dissolved in 50 μL TE buffer and stored at -20°C.

3.3 Quantification of DNA

To determine the concentration of the extracted DNA two methods was employed:

3.3.1. Analysis of Extracted DNA using Agarose gel electrophoresis

In extracted DNA 1% agarose gel was used. To prepare 1% agarose gel 0.5g agarose was weighed and added to 50mL of 1X TAE (Tris Acetate Buffer) pH 8.3 and subjected to heating in a microwave till agarose was completely dissolved. Once it cooled off to 60°C, 5 μL (10mg/ml) DNA intercalating fluorescent dye ethidium bromide is added to it and the gel was poured into the caster. The comb was put place to make wells and the caster was placed in the gel tank. TAE buffer was poured in the reservoirs of the electrophoresis tank. 2µl DNA dilution was taken from the 50µl stock along with The gel was run by 80mA for half an hour. This method was employed for the estimation of quantity of Genomic DNA in agarose and simultaneously assessing its integrity. The resulting gel is visualized under Ultra Violet Radiations and photographed using Gel Doc (Gel documentation system).

3.3.2 Optical Density DNA Quantification

The extracted DNA was quantified by measuring optical density OD at two wavelengths, 260nm and 280nm by employing a spectrophotometer. As a principle, Optical Density of DNA observed at 260nm is by default twice that of optical density observed at 280nm in case of pure extracted DNA with no protein contaminations. If the observed OD of extracted DNA 260:OD280 ratio turns out to be lesser that 1.8 it indicates the contamination of extracted DNA with protein residues. However, this can be rectified by phenol chloroform treatment.

3.4 Amplification of beta globin (Housekeeping Gene)

Quantitative PCR was performed by protocol designed by (Daniel et al. 2011). The total volume of reaction mixture was 12.5 μL per tube and its constituents were enzyme Taq Polymerase buffer and 2M of DNTPs, F and R primers (20 p mole each), thermo stable Polymerase(1U) and 1 μL DNA as template. To make the final volume of 12.5 µL, nuclease free water was added. The sequences of respective primers are enlisted in the table below.

Table 3.1 Primer Sequence for the amplification of β-globin gene. (Huang *et al,* 1988)

Serial No.	Code of Primer	Primer Sequence (5'----- 3')	Tm
1.	β-globin–F	ACACAACTGTGTTCACTAGC	58.4 °C
2.	β-globin–R	CAACTTCATCCACGTTCACC	60.4 °C

Standard PCR reaction was performed in thermo cycler (swift maxi-Esco MaxPro thermal Cycler). The integrity of DNA was assessed by amplifying the 110 bp fragment of the b-globin gene under the PCR profile; denaturing was done at 95◦C for 40 seconds, annealing was done at 58◦C for 40 seconds, and extension was done at 72C for 50 seconds for 35 cycles and a final extension at 72 ◦C for 7 minutes for a total 35 cycles.

3.4.1 Analysis of the respective PCR Product via Electrophoresis

To visualize and detect the presence of 110 bp fragment of beta globin DNA in acute appendicitis samples as well as control samples a 2% agarose gel was prepared. In order to detect the presence of EBV DNA fragment 12.5 µL of PCR product was loaded in the well with 1 X loading dye. These samples were run at 75 mA for 30 minutes. The resulting gel is visualized under the UV Light and photographed by Gel Documentation system.

3.5 Amplification of EBV DNA Using EBER Primers

In order to detect the EBV DNA in acute appendicitis samples along with control DNA samples the below mentioned PCR profile was used following standard PCR protocol to amplify the 170bp EBV DNA fragment. The constituents of the reaction mixture were MgCl2 (2mM), Taq polymerase buffer (1 X), EBER2F/ EBER2R (each used in 10 pmole), 2mM dNTPs (dATP, dTTP, dGTP, dCTP), Thermostable Taq polymerase (1 U) and 1 μL of DNA template To amplify EBV DNA in acute appendicitis samples, the primers designed targeted the EBER 2 region of EBV DNA, the attributes are enlisted in table 3.3.

Table 3.2 Sequences of primers used for amplifying EBV DNA fragment. (Nanbo *et al.*, 2002)

Serial No.	Code of Priner	Primer Sequence (5'----- 3')	Tm
1.	EBER2-F	AGG ACA GCC GTT GCC CTA GTG	58.3 °C
2.	EBER2-R	TAG CGG ACA AGC CGA ATA CCC T	56.7 °C

Standard PCR protocol was performed using a thermocycler. The 170 bp EBV DNA fragment was amplified at 51 °C annealing temperature. The optimized PCR profile used for primers EBER2F/EBER2R involved : denaturing for 40 secs at 95°C

3.5.1 Analysis of PCR Product by Agarose Gel Electrophoresis

To visualize and detect the presence of 170bp fragment of EBV DNA in acute appendicitis samples as well as control samples a 2% agarose gel was prepared. In order to detect the presence of EBV DNA fragment 12.5 μL of PCR product was loaded in the well with 1 X loading dye. These samples were run at 75 mA for half an hour. The result is visualized under the UV Light.

3.6 Amplification of MMTV Using LTR Primers

In order to detect the MMTV DNA in acute appendicitis samples along with control DNA samples the above mentioned PCR profile was used following standard PCR protocol to amplify the 630 bp EBV DNA fragment. The constituents of the reaction mixture were MgCl2 (2mM), Taq polymerase buffer (1 X), LTR 5 and 3 (each used in 2 0 pmole), 2mM dNTPs (dATP, dTTP, dGTP, dCTP), To amplify MMTV DNA in acute appendicitis samples, the primers designed targeted the LTR regions of MMTV their attributes are enlisted in table 3.3.

Table 3.3 Sequences of primers used for amplifying MMTV DNA fragment.

Serial No.	Primer Code	Primer Sequence (5'----- 3')	Tm
1.	LTR-5	GGT GGC AAC CAG GGA CTT AT	57.3 °C
2.	LTR-3	CGT GTG TTT GTG TCT GTT CG	54.5°C

Standard PCR protocol was performed using a thermocycler. The 630 bp MMTV DNA fragment was amplified at 50 °C annealing temperature. The optimized PCR profile used for primers LTR-5/LTR-3 involved : denaturing for 40 secs at 95°C .

3.6.2 Analysis of PCR Product by Agarose Gel Electrophoresis

To visualize and detect the presence of 630 bp fragment of MMTV DNA in acute appendicitis samples as well as control samples a 2% agarose gel was prepared. These samples were run at 100 Volts for 30 minutes. The resulting gel is visualized under the UV light and photographed by Gel Documentation system.

3.7 Detection of CMV DNA Using HCMV primers

To ensure that the integrity of the extracted DNA was fit human polymerase chain reaction for glycoprotein B of HCMV was performed. The 12.5ul PCR reaction mixture was made, composed of Taq Polymerase buffer(1X), MgCl2 (2mM), 2mM of DNTPs, forward and reverse primers(20 pmole each), thermos table Taq Polymerase(1U) and 1ul DNA template. The nuclease free water was added to make the final volume of 12.5ul.

To amplify HCMV DNA in acute appendicitis samples, the primers designed against the glycoprotein B region of HCMV.

Table3.4 Sequences of HCMV primers for amplifying CMV DNA fragment.

Serial No.	Primer Code	Primer Sequence (5'----- 3')	Tm
1.	HCMV-F	GGT GGC AAC CAG GGA CTT AT	58°C
2.	HCMV-R	CGT GTG TTT GTG TCT GTT CG	54.5°C

Standard PCR protocol was performed using a thermocycler. The 266 bp HCMV DNA fragment was amplified at 60 °C annealing temperature. The optimized PCR profile used for primers HCMV-F/HCMV-R involved: denaturing for 40 secs at 95°C , annealing at 60 °C for 45 sec, and an extension at 72°C for 40 seconds, 35 cycles in total ; and a final extension at 72°C for 10 min.

3.7.2 Analysis of PCR Product by Agarose Gel Electrophoresis

To visualize and detect the presence of 266 bp fragment of HCMV DNA in acute appendicitis samples as well as control samples a 2% agarose gel was prepared. These samples were run at 100 Volts for 30 minutes. The resulting gel is visualized under the UV light and photographed by Gel Documentation system.

3.8 Statistical Analysis of Data

For the analysis of the obtained results, 16.0 version of Statistical Package for the Social Sciences (SPSS) was used. Furthermore, the infection of CMV, MMTV and EBV DNA was linked with appendicitis samples. Also, the results for co infections of MMTV, CMV and EBV were recorded. Gender prevalence and age based distribution of appendicitis in patients were also analysed.

CHAPTER 4

RESULTS

4.1 Data Collection Acute Appendicitis and Control Samples

4.1.1 Clinical Data for Acute Appendicitis Blood Samples

For this study total 30 Acute appendicitis blood samples were obtained. The pie chart in figure 4.1 shows gender prevalence of acute appendicitis in patients from whom blood samples were obtained. 63% samples were from males and 37% samples were taken from females.

The patients ranged from 10 to 70 years of age. The bar chart shown in figure 4.2 shows that the probability of developing acute appendicitis is inversely proportional with age. Most of the reported cases are 10 to 20 years old.

A total of 30 control samples were collected for this study, 19 samples were taken from males while 11 blood samples were taken from females. The ages of the controls were matched to the samples with acute appendicitis.

4.2. Gender Prevalence of Acute Appendicitis

A total of 30 patients participated in the study by giving blood samples. Out of these participants 19 were male and 11 were female. The data was collected to establish whether acute appendicitis is prevalent in one gender compared to another. The following pie chart was made. The pie chart shows that 63% of the individuals suffering from Acute Appendicitis are males and 37%are females. **Table 4.1:** Gender prevalence of Acute Appendicitis

Gender	**Male**	**Female**
Number of Samples	19	11

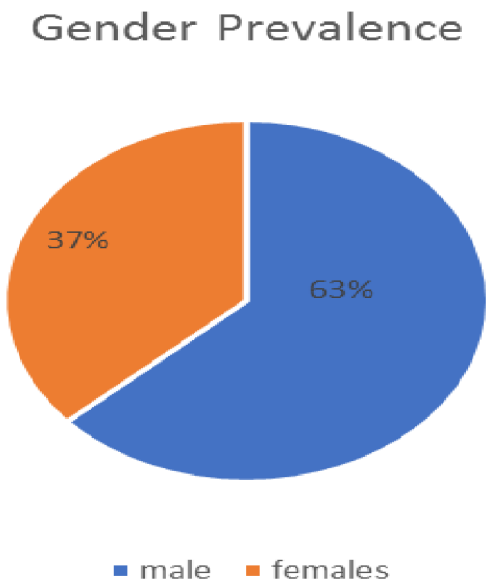

Figure 4.1: Gender prevalence of Acute Appendicitis

4.3. Association of Age with Acute Appendicitis

During sampling, the age of patients was recorded in the patient history form to determine whether an association exists between Acute Appendicitis and a certain age. Out of the total 30 subjects 12 were between 10-25 years, 10 were between 25-40 years, 7 were between 40-55 years and 1 was between 55-70 years.

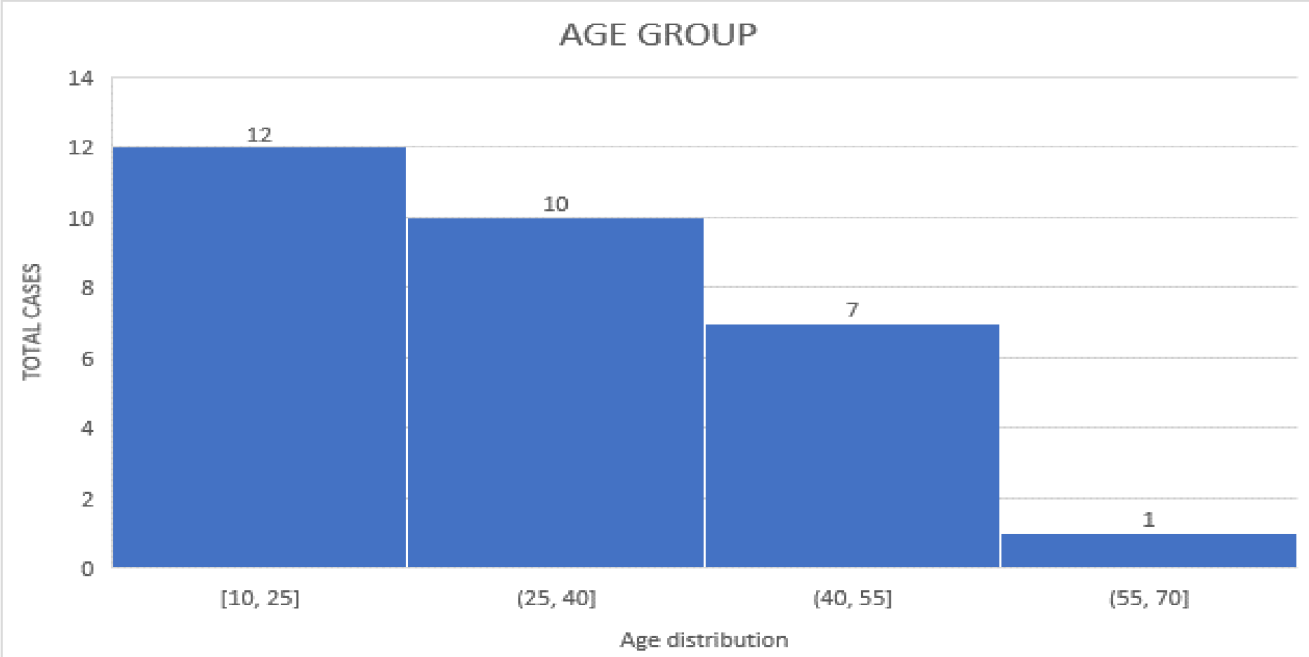

Figure 4.2: Age distribution of Acute Appendicitis

4.4 Beta Globin gene Screening

To check the integrity and quality of the extracted DNA beta globin gene is detected through PCR. The presence of a 110bp beta globin housekeeping gene fragment confirms the presence of DNA. Moreover, it shows that the DNA has a good quality and integrity for PCR.

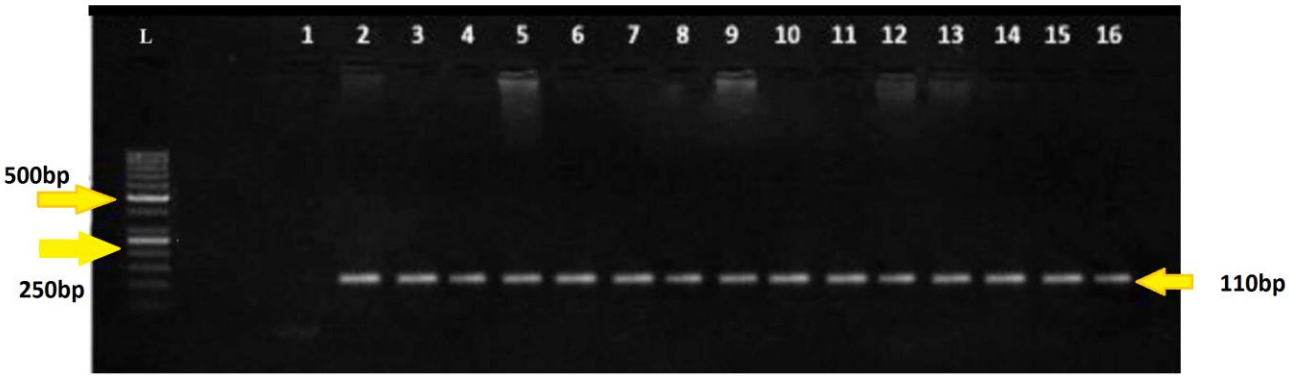

Figure 4.2 Amplification of 110 bp Beta globin gene product through PCR. Lane L has a 50bp DNA marker (Fermentas). Lane 1 has negative control. Lane 2 has positive control. Lane 3-16 has beta globin gene fragments amplified from extracted DNA blood samples of acute appendicitis.

4.5 Screening of Acute Appendicitis Samples for EBV EBER gene

Epstein–Barr virus-encoded are the non-coding RNAs. They are present in the EBV infected humans. PCR product for EBER based primers were used to detect EBV DNA in acute appendicitis patients. The amplified product of EBER gene fragment is 170bp.

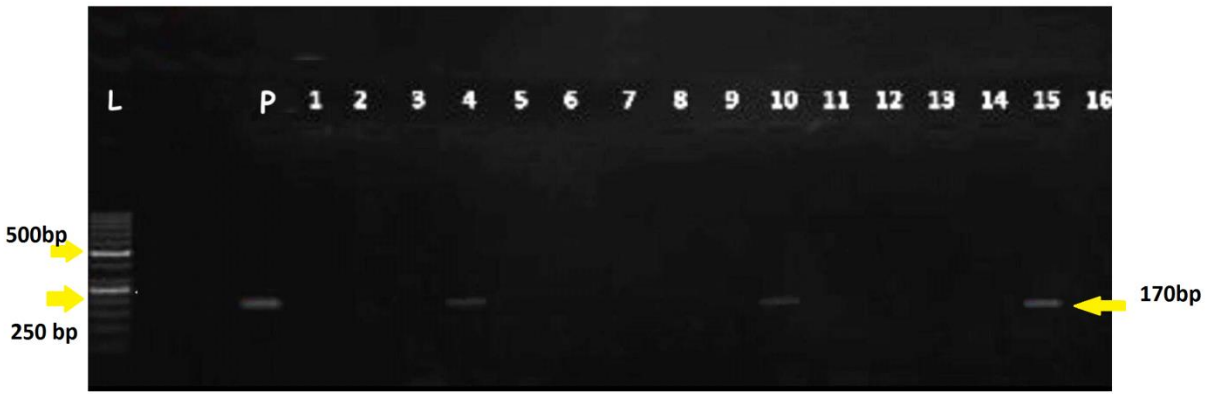

Figure 4.3 Amplification of 170 bp EBER gene product through PCR. Lane L has a 50bp DNA marker (Fermentas). Lane P shows positive control. Lane 1 shows negative control. Lanes 4, 10 and 15 shows EBV positive samples amplified for EBER gene in blood samples of acute appendicitis.

4.6 Screening of Acute Appendicitis Samples for MMTV LTRs

PCR fragment of MMTV LTRs is 630bp in size. The DNA extracted from blood samples of patients of acute appendicitis was screened for MMTV LTRs.

Figure 4.4 Amplification of 630bp MMTV LTR product through PCR. Lane M has a 100bp DNA marker (Fermentas). Lane P shows positive control. Lane 1 shows negative control. Lanes 7 shows positive sample showing amplified MMTV LTR in blood samples of acute appendicitis.

4.7 Screening of Acute Appendicitis Samples for HCMV

PCR fragment of HCMV is 266 bp in size. The DNA extracted from blood samples of patients of acute appendicitis was screened for HCMV.

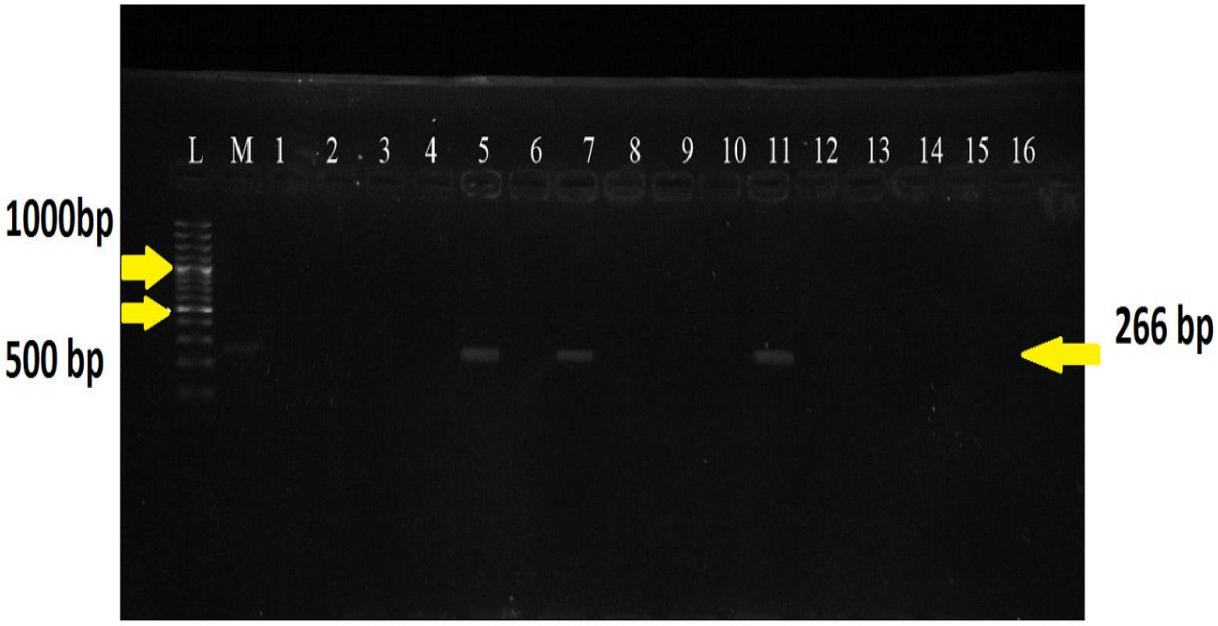

Figure 4.5 Amplification of 266 bp HCMV product through PCR. Lane M has a 100bp DNA marker (Fermentas). Lane P shows positive control. Lane 1 shows negative control. Lanes 7 shows positive sample showing amplified HCMV in blood samples of acute appendicitis.

4.8 Prevalence of CMV, EBV and MMTV in Acute Appendicitis

The table 4.2 shows the frequency table shows prevalence of viruses EBV, CMV and MMTV. MMTV was positive in 1 sample, CMV in 4 samples and EBV in 3 samples of acute appendicitis out of 30. None of the control samples was positive for CMV, EBV or MMTV.

Table 4.2: Prevalence of CMV, EBV and MMTV in acute appendicitis

Sr No	Screened Virus	Total Acute Appendicitis Samples	Positive Samples	Prevalence
1	CMV	30	4	13%
2	EBV	30	3	10%
3	MMTV	30	1	1%

4.9 Frequency table for Mono-infection and Co-infection Prevalence

The graph in figure 4.6 shows the frequency table results for prevalences of viruses. Monoinfections of EBV, CMV, MMTV were seen in 27% (8/30) samples. Co infections were seen in 3% (1/30) samples. And no infections were seen in 73% (22/30) samples of acute appendicitis.

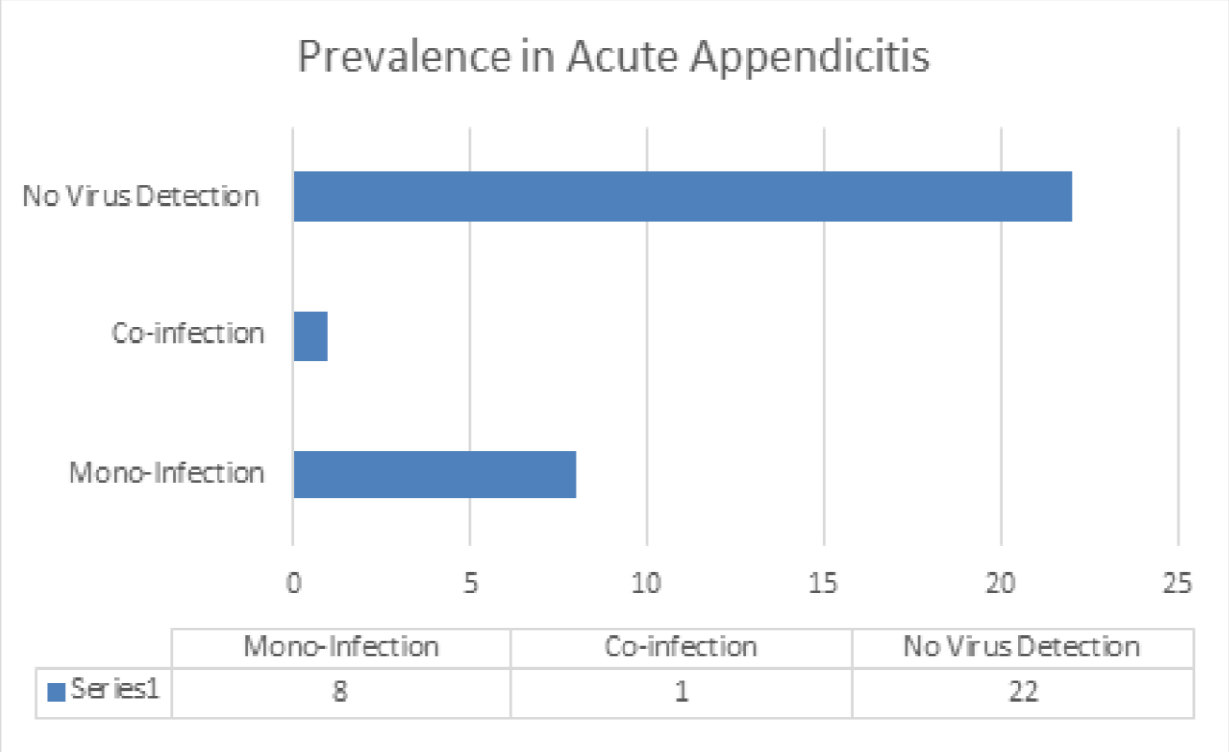

Figure 4.6: Prevalence of Mono and coinfection of MMTV, CMV and EBV in Acute Appendicitis samples.

CHAPTER 5

DISCUSSION

Appendicitis is a common clinical condition defined as the inflammation of the appendix (Graffer *et al.,* 1996). The appendix - a part of the gastrointestinal tract, is a 3 1/2-inch blind tube of tissue that extends from the large intestine. The role of appendix in humans has been elusive and undefined and its removal does not have any bad consequences. However, the inflammation of the appendix in humans has severe consequences and results in death (Hobler *et al*., 1996).

About 250,000 cases of acute appendicitis are reported in The United States and 40,000 in the United Kingdom annually. Acute appendicitis causes considerable mortality and morbidity. In 2013 about 16 million cases of appendicitis occurred. This resulted in 72,000 deaths globally (Weir *et al.,* 2013).

Acute Appendicitis is reported to have a high incidence in Pakistan. The ultimate treatment that healthcare professionals resort to is open or laparoscopic appendectomy. Studies like this have the potential to fulfil the etiological gap of acute appendicitis and to prove if a pathogen of viruses related pathogenesis of acute appendicitis exists. Previously, very little research has been done to speculate and confirm the role of viruses in the etiology and pathogenesis of acute appendicitis If the role of viruses in causing acute appendicitis is confirmed then novel viral based diagnostic tests will be designed for acute appendicitis. Moreover, acute appendicitis will be cured by antiviral therapy and there will be no need for a surgical appendectomy (Graffer *et al.,* 1996).

Acute appendicitis is the primary cause of abdominal surgery in and it is diagnosed in 1-

8% of children inflicted with abdominal pain (Katzoli *et al.,* 2004). Acute Appendicitis is manifested by , vomiting, anorexia, loss of weight as a consequence of loss of appetite, periumbilical pain, nausea, right quadrant lower quadrant pain, swelling of the abdomen, constipation, diarrhea, flatulence, severe abdominal cramps and fever. Diagnostic difficulties of acute appendicitis lead to perforations of appendix in 30- 60% cases (Addis *et al.,* 2004) .

In the present study, we verified that EBV, CMV and MMTV viral sequences maybe present in the host DNA as mono- infection co-infection. None of the viruses was detected in the gender and age matched healthy control samples.

Even though acute appendicitis has a high incidence worldwide, its etiology is still largely unknown. Various theories have been considered over time however; none of them have been scientifically proven. No evidence has previously proven the possibility of a pathogen or virus related etiology of acute appendicitis. Therefore, this study was designed to study the detect the presence of EBV, CMV and MMTV in 30 patients of acute appendicitis that underwent an appendectomy. Moreover, gender prevalence and age based prevalence of acute appendicitis was also studied.

Cytomegalovirus was the most common virus in our study. 4 out of 30 samples were positive for cytomegalovirus showing a 13.3% prevalence. Katzoli et al reported a study with 21% incidence of Cytomegalovirus in patients of acute appendicitis, making it the herpes virus with the highest incidence in acute appendicitis

samples in that study. In another study CMV has been implicated in ulcerative colitis and appendicitis (Sylvie *et al.,* 2013). Similarly, Zakanafani *et al* reported that acute appendicitis developed in an immunocompetent patient following a Cytomegalovirus and EBV infection.

Epstein Barr Virus had the second highest incidence in acute appendicitis patients in this study. 3 out of 30 samples were infected with showing a 10% prevalence. Katzoli *et al* reported a study with 7% incidence of EBV in patients of acute appendicitis. It was reported that acute appendicitis developed after an EBV infection in an immunocompetent patient (Za Kanafani *et al.,* 2004). In various studies conducted it has been reported that Epstein-Barr Virus Infection is commonly reported in Inflamed gastrointestinal Mucosa (Julie *et al.,* 2013).

Contrary to our findings, studies conducted previously report a coinfection of EBV and CMV in patients suffering from acute appendicitis. In our study, none of the samples were co-infected with EBV and CMV. However, one sample was infected with CMV as well as EBV. This co-infection has not been reported by any previous studies. Out of 30 samples 22 were not infected by any of the viruses.

According to our findings MMTV had the least incidence i.e. only 1 out of 30 samples was positive for MMTV having a 3.3 percentage. No significant association was found between MMTV and Acute appendicitis in this study. No previous study has been conducted to find an association between MMTV and acute appendicitis. However,

the pro-inflammatory nature of MMTV was demonstrated by a study conducted by Fernandez et al which showed that the MCF-7 cell line containing the MMTV envelope showed an increased expression of TGF and TNF genes that are responsible for causing inflammation in cells in comparison to the other subtype which did not contain MCF-7 subtype (Fernandez *et al.,*2006).

In our study, out of the 30 acute appendicitis participants 19 were male and 11 were female. Hence, 63% of the individuals suffering from Acute Appendicitis are males and 37% are females. This finding was consistent with previous one which show that acute

appendicitis is more prevalent in males compared to females. One particular study had a 1.3:1 ratio of males to females with acute appendicitis (Richardson *et al.*,2011)

According to the findings of this study, acute appendicitis incidence shows a decrease with an increase in age. Out of the total 30 samples 12 are between 10-25 years, 10 are between 20-45 years, 7 are between 40-55 years and 1 is between 55-70 years. This finding is consistent with previous studies which state that appendicitis is most common in children between 10-17 years of age (Bratton *et al.*, 2000). Another study states that in children subjected to appendectomies 77% range from 6-18 years in age (Addis *et al.*,1990)

CMV, MMTV lead to the inflammation or the inflammation lead to the release of pathogens. Hence, studies conducted to find out the direct and detailed association of viruses with acute appendicitis randomized and prospective studies need to be conducted with a larger sample size.

CONCLUSION & FUTURE PROSPECTS

CMV was positive in 13%, (4/30) samples EBV were positive in 10 %(3/30) and MMTV in 3 % (1/30) of the Pakistani acute appendicitis samples respectively. The significant percentage of prevalence showed that CMV and EBV may potentially have a role in developing acute appendicitis. However, further prospective studies are required to elucidate whether a direct association exists between these viruses and acute appendicitis In conclusion, the development of acute appendicitis is multifactorial and may not be caused by a single virus or a single event. Nonetheless, we can conclude from our data that EBV and CMV may be responsible for triggering acute appendicitis. Significant correlations found in parameters like patients age and gender with acute appendicitis. Hence, this study imparts valuable information to the existing information present regarding viral etiology of acute appendicitis and supplements the findings of previous studies and research groups. To confirm the findings of this study furthermore prospective studies with larger sample sizes need to be conducted in the future.

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