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"Molecular epidemiology of Adenovirus circulating in southern Palestine"

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Dedication

My Dear Husband, Father, Mother, Brothers and Sisters

For their Patience, Encouragement and Support

With love and Respect

Declaration:

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis or any part of the same has not been submitted for a higher degree to any other university or institution.

Signed:

Lina Salah Salem Qurei

Date:

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Abstract

Three hundreds and thirty eight nasopharyngeal aspirates from children with Adenovirus respiratory tract infection admitted to the caritas baby hospital between January 2005 and Dec 2010 were studied in this work. All children were residing southern Palestine; districts Hebron and Bethlehem. The epidemiological characteristics of Adenovirus were investigated by significant test results (one sample test for association and Pearson test for correlation) in detail by examination of 95% confidence intervals for each category. In terms of residency, most children with adenoviral infections were from villages (68%), in terms of age distribution, most cases were among infants (62%), in terms of sex distribution, most cases were among males (62%). Regarding seasonal occurrence, the largest number of Adenoviral infections occurred during winter and spring months. Most infants and children diagnosed with Adenovirus respiratory tract infection suffered mainly from Bronchitis, Bronchopneumonia (33.3%) and upper respiratory tract infections (URTI, 24.7%). The major symptom found in 80% of children was fever, followed by cough, found in 50% of the children. Despite etiology of the viral infection, 70% of children had been treated with antibiotic. Utilizing genetic analysis of the samples, different sizes of hexon genes were detected via polymerase chain reaction (PCR). Sequence analysis of the samples' DNA revealed that different Adenovirus serotypes were circulating in southern Palestine, mainly; HADV-1, HADV-2, HADV-3 and HADV-5. The most frequent serotypes however, were HADV-1, HADV-2 and HADV-3. Precise alignment and Phylogenetic analysis generated by neighbor-joining method of Palestinian sample's amino acids were in agreement with the DNA analysis. Surprisingly, some Palestinian serotypes were found to be homolog for two different reference HADV serotypes simultaneously, suggesting that they may be representing new unpublished serotypes. Interestingly, the different serotypes found in southern Palestine did not correlate specifically to residency, age or sex of the diseased children. The most striking finding was that some serotypes correlated strongly to specific seasons, rather than others. While serotypes HADV-2 and HADV-3 occurred mainly in the spring months, HADV-1 and HADV-5 were found predominantly in the winter months.

In conclusion, applying molecular Biology based methodology for detection of infectious diseases in combination with epidemiological data is becoming an urgent and essential approach in developing strategies for control and treatment of viral infections.

الملخص

كان عدد العينات الماخوذة من المجاري التنفسية كالحلق والانف للاطفال الذين دخلوا الى مشفى الكريتاس بفيروس الادينو هو 338، وهو فيروس يصيب بشكل عام القناة التنفسية خاصة بين الاطفال. جرت الدراسة في الفترة الممتدة بين كانون اول2005-2010 كانون ثاني, وكان جميع الاطفال الذين تم شملهم في الدراسة من منطقة جنوب فلسطين اي الخليل وبيت لحم. الخصائص الوبائية لفيروس الادينو تم فحصها عن طريق برنامج التحليل الاحصائي بواسطة مقياس الثقة CI 95% لكل عامل من عوامل المرض (one sample test for association and Pearson test for correlation). بالنسبة لمكان الاقامة كانت اكثر الحالات انتشارا بفيروس الادينو هو بين الاطفال الذين يسكنون القرى بنسبة 68%. اما من ناحية التوزيع العمري للاطفال المصابين بالفيروس كانت اكثر الحالات بين الاطفال دون عمر السنة وهي 62%. اما بالنسبة للتوزيع حسب الجنس كانت نسبة الذكورالمصابين اعلى من الاناث حيث كانت النسبة بين الذكور تساوي 62%. من حيث حدوث المرض بالنسبه للاشهر فكان اعلى حدوث للاصابة بالفيروس في فصل الشتاء واشهر الربيع. من الامور الهامة التي لوحظت على حديثي الولادة والاطفال المصابين بالفيروس انهم كانوا يعانون بشكل رئيسي من التهابات رئوية والتهابات في الشعب الهوائية بنسبة تساوي 33.3% وكانوا يعانون من التهابات في المجاري التنفسية العليا بنسبة تساوي 24.7%. اما عن اهم الاعراض المصاحبة لفيروس الادينو فكانت الحمى بنسبة تساوي 80% والسعال بنسبة تساوي 50%. اما على صعيد العلاج فقد تم اعطاء 70% من الاطفال الذين دخلو الى المشفى وكان لديهم فيروس الادينو مضادات حيوية.

تم تحليل جين Hexon الذي يحتوي على كل الاختلافات للسلالات الجينية للفيروس. تم تحديد هذه التنوعات عن طريق الـ PCR لِـ DNA لكل عينة لمعرفة السلالات المختلفة للفيروس وحجمها. تحليل السلالات الجينية لفيروس الادينو الموجودة في جنوب فلسطين دلت على وجود HADV-1, HADV-2,HADV-3 ,HADV-5 ولكن اكثر السلالات انتشارا كانت HADV-1, HADV-2,HADV-3. المطابَقة والشجرة الجينية التحليلية بنيت على اساس طريقة تسمى neighbor-joining للحمض النووي والحمض الاميني لمنطقة جنوب فلسطين. من المدهش ان بعض السلالات في فلسطين وجدت مطابقة لسلالتين في نفس الوقت من فيروس الادينو المكتشفة سابقا وهذا الامر يمكن ان يقود الى ايجاد سلالات جديدة غير موجودة او غير معرّفة من قبل. السلالات المختلفة التي وجدناها في جنوب فلسطين لا ترتبط بالتوزيع الجغرافي والعمر والجنس للاطفال المصابين ولكن بعض السلالات ترتبط بشكل قوي مع اشهر معينة اكثر من غيرها، بينما الاصابات بِـ HADV-2,HADV-3 حدثت بشكل رئيسي في اشهر الربيع اما الاصابات بِـ HADV-5 و-HADV 1 فكان في اشهر الشتاء.

الخلاصة: تطبيق علم الاوبئة والاحياء الجزيئية هو اساس في تحديد الامراض المعدية واصبح هاما وضروريا لتطوير استراتيجيات للسيطرة على انتشار المرض وايجاد العلاج للاصابات والالتهابات الفيروسية.

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1. Introduction

1.1 Respiratory tract infections and common cold

Respiratory tract infections are a major cause of human morbidity, and are caused by a broad spectrum of microbial agents. Upper respiratory tract infections are a leading cause of time lost from work and school, consuming large amounts of health care financial resources (Allander et al., 2005). Viruses account for the largest number of respiratory tract infections.Bacteria account for up to 25 percent of upper respiratory tract infections (Goldmann et al., 2003). The so-called respiratory viruses include influenza virus A and B, parainfluenza viruses, Adenoviruses, respiratory syncytial virus (RSV), rhinoviruses, and corona viruses. In recent years, several novel viruses have been discovered in patients with respiratory infections using molecular biology methods (Weissbrich et al., 2006). These novel viruses include the human metapneumovirus and several corona viruses (SARS, NL63, and HKU1) (Fouchier et al., 2005). The latest addition to this list was the human Boca virus (hBoV) described by Allander et al. (Allander et al., 2005).

Rhinoviruses and corona viruses together cause more than 50 % of "colds." The common cold (viral upper respiratory tract infection; VURTI, acute viral Rhinopharyngitis, acute coryza, or cold) is a contagious, viral infectious disease of the upper respiratory system, caused primarily by rhinoviruses and corona viruses (Arden and Mackay, 2010). Typical symptoms include cough, sore throat, rhino rhea (runny nose), and fever. The common cold is the most frequent infectious disease in humans with an average of two to four infections a year in individual adults and up to 6–12 infections per year in children. Collectively, colds, influenza, and other infections with similar symptoms are included in the diagnosis of influenza-like illness and are also termed "upper respiratory tract infections" (URTI). Influenza involves infection of the lungs while the common cold does not (Olenec et al., 2010). Common colds are droplet-borne infections, which means that they are primarily transmitted through breathing in tiny particles that the infected person emits when he or she coughs, sneezes, or exhales.

1.1.1 Influenza

Influenza viruses are classic respiratory viruses and cause endemic, epidemic and pandemic influenza. There are three types of influenza virus: A, B, and C. Influenza A viruses causes epidemics and occasionally pandemics, and there is an animal reservoir, notably in birds. Influenza B viruses only cause epidemics and do not involve animal hosts. Influenza C viruses do not cause epidemics and give rise to only minor respiratory illness (Mims, 2004).

1.1.2 Parainfluenza virus (HPIV)

HPIVs primarily affect young children and the pathogenic spectrum includes upper and lower respiratory tract infections. HPIVs are responsible for 30%-40% of all acute respiratory tract infections in infants and children. These conditions include common colds with fever, croup, bronchiolitis, and pneumonia. HPIVs also cause community-acquired respiratory tract infections of variable severity in infants **(**Teo et al., 2010).

1.1.3 Human respiratory syncytial virus (RSV)

(RSV) is a virus that causes respiratory tract infections. It is the major cause of lower respiratory tract infection and hospital visits during infancy and childhood. 75 % of bronchiolitis infections are caused by respiratory syncytial virus (Mims, 2004). There is no vaccine. Treatment is limited to supportive care, including oxygen. In temperate climates there is an annual epidemic during the winter months. In tropical climates, infection is most common during the rainy season (Hall et al., 2009).

1.1.4 Metapneumovirus (HMPV)

HMPV is a respiratory pathogen closely related to RSV and is thought to occur mostly in the winter months. It is associated with a spectrum of illness from mild infection to bronchiolitis and pneumonia (Mims, 2004).

1.1.5 Coxackie and Bocaviruses

The most well known Coxsackie A disease is hand, foot , and mouth disease (unrelated to foot and mouth disease), a common childhood illness which affects mostly children aged 10 or under, often produced by Coxsackie A16 (Rabenau et al., 2010). In most cases, infection is asymptomatic or causes only mild symptoms. In others, infection produces short-lived (7–10 days) fever and painful blisters in the mouth (a condition known as *herpangia*), on the palms and fingers of the hand, or on the soles of the feet. There can also be blisters in the throat, or on or above the tonsils, and other components of Waldeyer's Ring. Adults can also be affected. The rash, which can appear several days after high temperature and painful sore throat, can be itchy and painful, especially on the hands/fingers and bottom of the feet (Rabenau et al., 2010).

Human Boca virus (HBoV) is a parvovirus, which causes lower respiratory tract infections. HBoV is found rarely in respiratory samples from healthy subjects. In patients with respiratory complaints, it can be found alone or, more often, in combination with other viruses known to cause respiratory complaints (Ricour and Goubau, 2008). Newborns are probably protected by passive immunization. Although the age group most frequently affected appears to be children between the ages of six months to two years (Endo et al., 2007), infection in children older than five and even in a 28-year-old have been reported (Kupfer et al., 2006).

1.1.6 Adenoviruses

1.1.6.1 History

Adenoviruses were first isolated and characterized as distinct viral agents by two groups who were searching for the etiologic agents of acute respiratory infections. In 1953, Rowe and colleagues observed the spontaneous degeneration of primary cell cultures derived from human adenoids (Knipe et al., 2001). The pathogenic changes proved to result from the replication of previously unidentified viruses present in the adenoid tissues. In 1954, Hilleman and Werner were studying an epidemic of respiratory disease in army recruits, and they isolated agents from respiratory secretions that induced cytopathic changes in cultures of human cells (McDonald et al., 1999).

In 1956, the agents were named *Adenoviruses,* after the original tissue (adenoid) in which the prototype viral strain was discovered (Knipe et al., 2001). Epidemiologic studies confirmed that adenoviruses are the cause of acute febrile respiratory disease among military recruits (Ginsberg et al., 1955). It soon became clear, however, that Adenoviruses are not the etiologic agents of the common cold; they are responsible for only a small portion of acute respiratory morbidity in the general population and for about 5% to 10% of respiratory illness in children. Besides respiratory disease, Adenoviruses cause epidemic conjunctivitis (Niemann et al., 1993), and they have been associated with a variety of additional clinical syndromes—perhaps most notably, infantile gastroenteritis (Parrott et al., 1954). Today, more than 100 members of the adenovirus group have been identified that infect a wide range of mammalian and avian hosts. All of these viruses contain a linear, double-stranded DNA genome encapsulated in an icosahedral protein shell (Polvino-Bodnar et al., 1987). In 1962, Trentin and colleagues made a seminal discovery: human adenovirus type 12 induces malignant tumors after inoculation into newborn hamsters. This was the first time that a human virus was shown to sponsor oncogenesis. No epidemiologic evidence has been reported linking Adenoviruses with malignant disease in humans; extensive searches have generally failed to find Adenovirus nucleic acids in human tumors (Turner et al.; 1993; Harmonet al., 1979). Nevertheless, the ability to induce tumors in animals and to transform cultured cells has established Adenovirus as an important model system for probing the mysteries of oncogenesis (Knipe et al., 2001). As the interest in adenoviruses as tumor viruses intensified, their virtues as an experimental system became evident (Knipe et al., 2001). The

prototype human adenoviruses are easily propagated to produce high-titer stocks, and they initiate synchronous infections of established cell lines (Harmon et al., 1979). Furthermore, the viral genome is readily manipulated, facilitating the study of adenovirus gene functions by mutational analysis (Harmon et al, 1979). Studies of adenovirus-infected cells have made numerous contributions to the understanding of viral and cellular gene expression and regulation, DNA replication, cell cycle control, and cellular growth regulation (Knipe et al., 2001).

1.1.6.2 Structure

Adenoviruses represent the largest nonenveloped viruses. Because of their large size, they are able to be transported through the endosome (i.e. envelope fusion is not necessary). The virion also has a unique "spike" or fiber associated with each penton base of the capsid that aids in attachment to the host cell via the coxsackie-adenovirus receptor on the surface of the host cells (Knipe et al., 2001; figure 1.1).

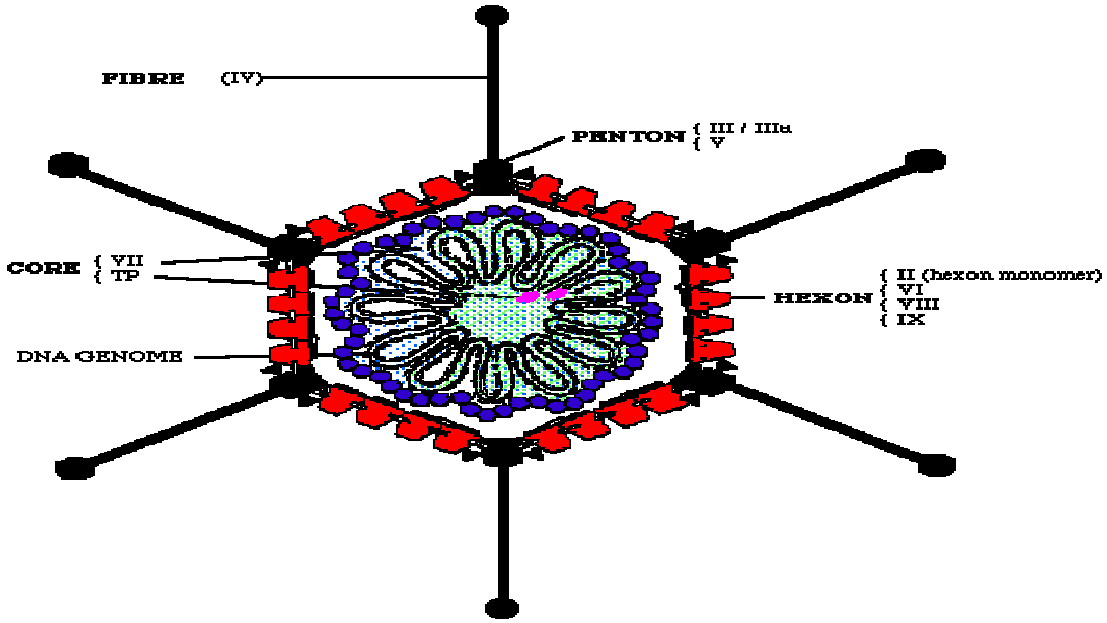

Figure 1.1: general structure of Adenovirus (Knipe et al., 2001).

1.1.6.3 Genome

The Adenovirus genome is a linear, non-segmented double stranded (ds) DNA which is between 26 and 45 Kbp. This allows the virus to theoretically carry 22 to 40 genes. Although this is significantly larger than other viruses in its group, it is still a very simple virus and is heavily reliant on the host cells for survival and replication. An interesting feature of this viral genome is that it has a terminal 55 kDa protein associated with each of the 5' ends of the linear dsDNA; these are used as primers in viral replication and ensure that the ends of the virus' linear genome are adequately replicated (Knipe et al., 2001).

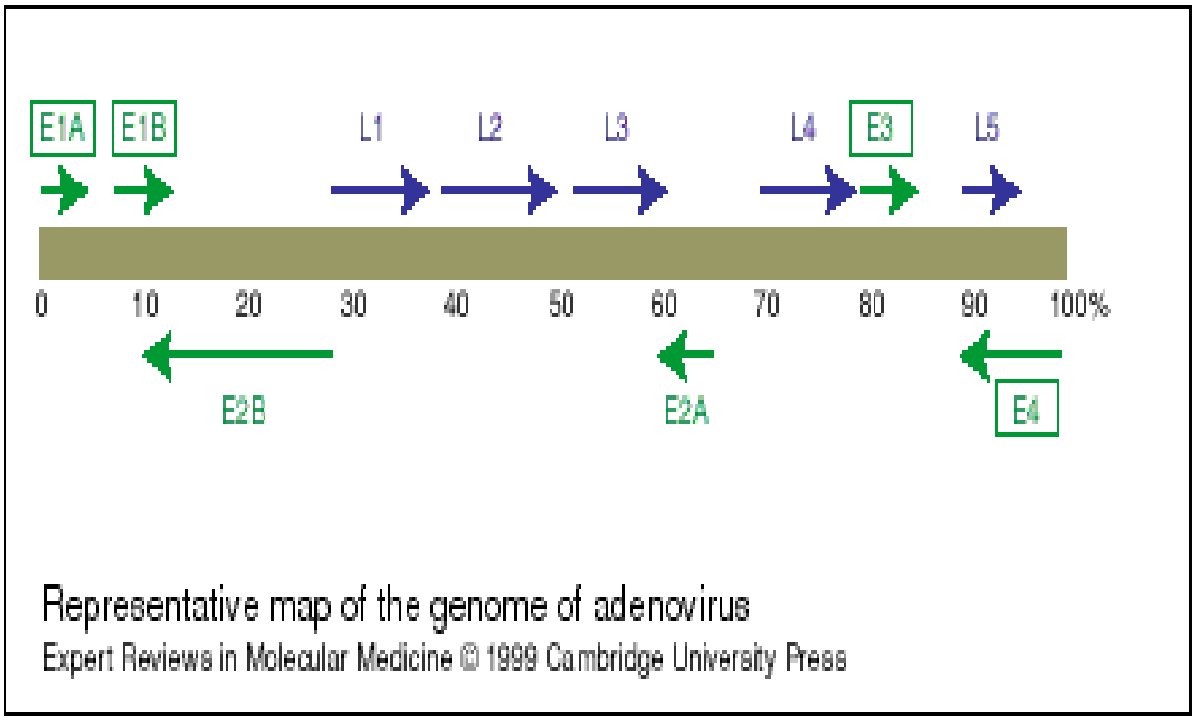

Figure 1.2: Schematic diagram of the linear Adenovirus genome, showing early genes (E) and late genes (L) **(Journals.cambridge.org)**.

The genes in boxes (figure 1.2) are those that can be removed during the production of a replication-defective virus for gene therapy protocols. The E1A gene (which encodes the initial viral transcription unit) must be removed to prevent the recombinant virus from replicating. Other genes can be deleted to make more space for the insertion of larger transgenes and, in the case of E4-deletion recombinant Adenoviruses; the immunogenicity has been reported to be significantly reduced **(Knipe et al., 2001).**

1.1.6.4 Classification

The Adenoviruses constitute the adenoviridae family of viruses, which is divided into two genera, Mastadenovirus and Aviadenovirus whereas the Aviadenovirus genus is limited to viruses of bird, the Mastadenovirus genus includes human, simian, murine, bovine, equine, porcine, ovine, canine, and opossum viruses. Although there is antigenic cross – reactivity among members within each genus owing to conserved epitopes located on the hexon protein of the virion, there is no known antigen common to all Adenoviruses (Knipe et al., 2001).

1.1.6.5 Serotypes

Human Adenoviruses (HADVs) include 53 recognized serotypes assigned to seven species (A-G) on the basis of biophysical, biochemical, and genetic criteria (Kajon et al., 2010). Serotype correlates with the severity and symptomology of disease, as well as with epidemiological characteristics (Metzgar et al., 2007). Knowledge of circulating serotypes can also predict the potential usefulness of available vaccines. The worst epidemics, often caused by newly emerging genome types may occur in areas where the causal Serotype was rare in recent times (Noda et al., 2002). Species B includes two genetic clusters, subspecies B1 and B2. Between 2010 and today, further 5 adenoviral serotypes were identified making the total serotypes 58 respectively (Ishiko and Aoki, 2009; Walsh et al., 2010; Robinson et al., 2011)

The serotypes of subspecies B1 (HADV-3, HADV-7, HADV-16, HADV-21, and HADVs- 50) generally cause acute respiratory disease (ARD) (Kajon et al 2010). HADV-C species includes serotypes 1, 2, 5 and 6. These serotypes are commonly associated with febrile respiratory illness in children and are noted to be endemic in certain regions (Metzgar et al., 2007). Species E (HADV4) are also frequent causative agents of epidemic conjunctivitis and respiratory disease (Metzgar et al., 2007). Recent respiratory outbreaks of a virulent strain of human Adenovirus B14 (HAdV-B14) led to the deaths of nine people in the United States during the 2006-2007 respiratory season (Yeung et al., 2009).

1.1.6.6Life cycle

The replication cycle is divided by convention into two phases that are separated by the onset of viral DNA replication (figure 1.3). The replication cycle include adsorption, penetration, movement of the viral DNA to the nucleus, and expression of an early set of genes (Knipe et al., 2001). Early viral gene products mediate further viral gene expression and DNA replication, induce cell cycle progression, block apoptosis, and antagonize a variety of host antiviral measures (Knipe et al., 2001). In HeLa cells infected at a multiplicity of 10 plaque-forming units per cell, the early phase lasts for 5 to 6 hours, after which viral DNA replication is first detected concomitant with the onset of viral DNA replication; the late phase of the cycle begins with expression of late viral genes and assembly of progeny virion. The infectious cycle is completed after 20 to 24 hours in HeLa cells. At the end of the cycle, about 10^4 progeny virus particles per cell have been produced, along with the synthesis of a substantial excess of virion proteins and DNA that are not assembled into virions. Cells infected at high multiplicity seldom divide; hence, at the completion of the replication cycle, the DNA and protein content of the infected cell has increased by a factor of about two (Knipe et al., 2001).

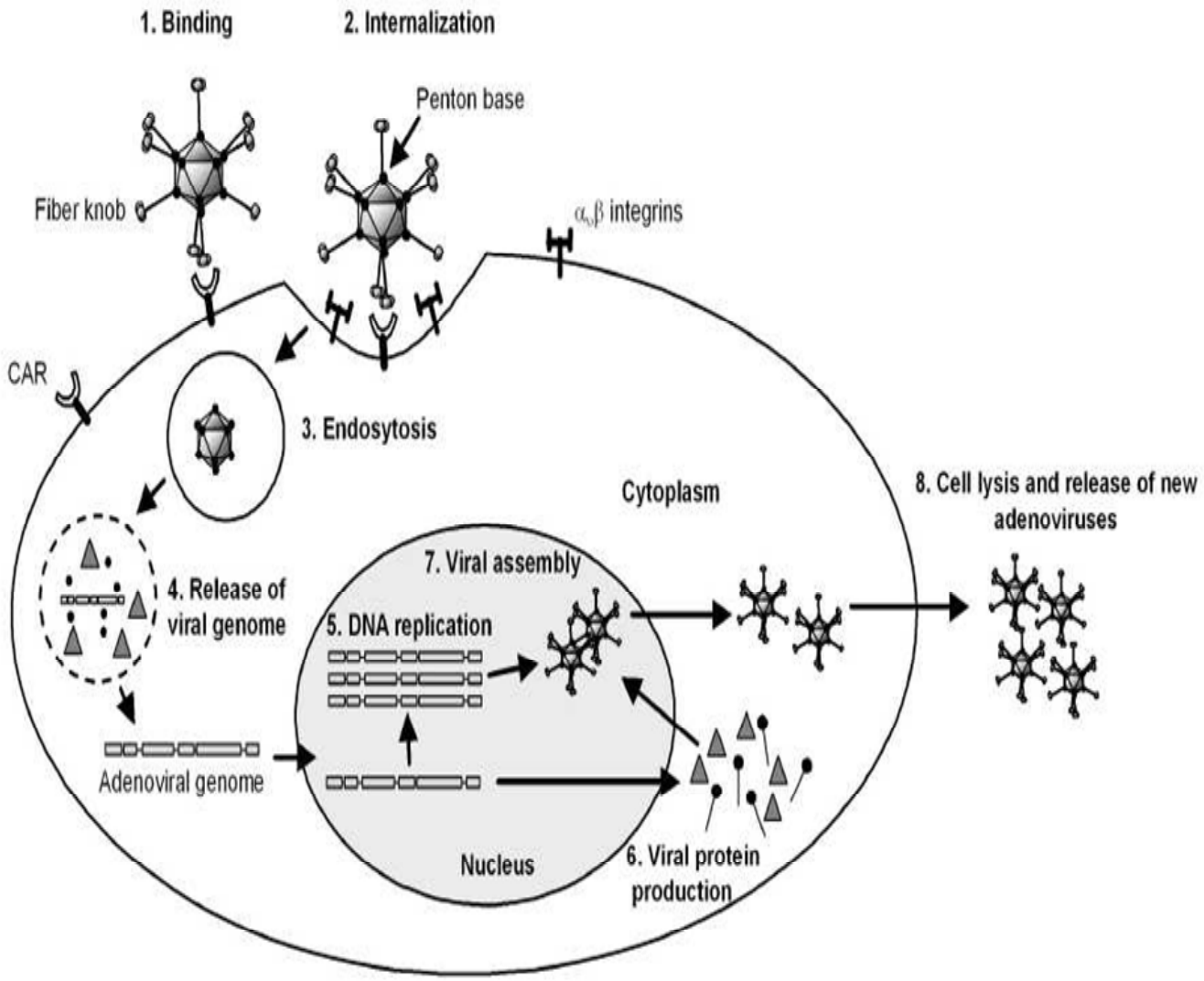

Figure 1.3: Schematic representation of Adenovirus life cycle (Microbiology 2009.wikispaces.com\Adenovirus+US).

Although early and late are convenient terms for description of events that occur during the replication cycle, the functional distinction between early and late events is often blurred. Early genes continue to be expressed at late times after infection, and the promoter controlling expression of the major late transcription unit directs a low level of transcription early after

infection. The viral genes encoding proteins IVa2 and IX begin to be expressed at an intermediate time and thus form a delayed-early category (Knipe et al., 2001).

1.1.6.7Clinical features

1.1.6.7.1 Acute Respiratory Disease

In many respects, ARD is similar to the description furnished earlier of the respiratory infection of children. The syndrome is caused by HADV4, HADV7, and occasionally HADV3. ARD is a syndrome that frequently occurs under the special conditions of fatigue and crowding created soon after the induction of young military recruits (Heubner et al., 1955). It is less common among healthy adults. Some cases have had a fatal outcome from the pneumonitis that may accompany and complicate the other, milder respiratory symptoms (Knipe et al., 2001).

1.1.6.7.2 Pertussis-like Syndrome

The association of Adenovirus infection with a pertussis-like syndrome has been noted, leading to some speculation that adenoviruses rather than *Bordetella pertussis* caused many of the cases of clinical whooping cough (Knipe et al., 2001). Claims were made that even the lymphocytosis of pertussis might be related to the Adenovirus infection (Collier et al., 1966). This point of view was given some credence by the isolation of an HADV5 from multiple organs of a patient with severe whooping cough with lymphocytosis that ended fatally (Collier et al., 1966). Later data from a controlled study of 134 children with a pertussis-like illness and 101 healthy controls report the common association of Adenoviruses with whooping cough symptoms; however, there is no evidence that the adenoviruses alone are responsible for the syndrome. The large number of Adenovirus isolates may be due to conditions favorable for reactivation of latent viruses from tonsillar tissue during concurrent *B.pertussis*infection(Knipe et al., 2001).

1.1.6.7.3 Infections of the Eye

An acute follicular conjunctivitis may occur as part of a respiratory-pharyngeal syndrome or as a separate entity (Bennett et al., 1957). Both bulbar and palpebral conjunctival involvement may occur and affect both eyes. The disease is often accompanied by significant preauricular lymphadenopathy (Bell et al., 1955). Complete recovery without sequelae is the most common result of this rather mild illness. The incubation period is usually 6 to 9 days, but it was as short as 2 days in experimental infections in volunteers (Knipe et al., 2001). Epidemiologically, these infections can occur sporadically or cause disease in large groups of contacts. Family members may be affected. When the source is a swimming pool or small lake, large numbers of children and young adults may develop symptoms. Swimming pool conjunctivitis is probably most commonly due to Adenoviruses (Knipe et al., 2001). Although the virus is isolated from the conjunctiva of affected individuals, it has not been isolated from water samples from putatively infected sources. The common-source water-borne outbreaks usually occur in summer and are caused by HADV3 and HADV7. However, other types, such as HADV1, HADV2, HADV4, HADV6, HADV9, HADV10, HADV11, HADV15, HADV16, HADV17, HADV20, and HADV22 (groups B, C, D, and E and HA groups I, II, and III) have been associated with this syndrome (Knipe et al., 2001).

1.1.6.7.4 Acute Hemorrhagic Cystitis

Acute hemorrhagic cystitis, an illness occurring almost exclusively in boys and associated with HADV11, HADV7, HADV35 and HADV21 is characterized by gross hematuria. Its significance lies in the potential confusion with other, more serious diseases of the kidney (such as glomerulonephritis). This self-limited disease is usually not accompanied by fever or

hypertension, and tests of renal excretory and concentrating functions have been essentially normal (Akiyama et al., 2001).

1.1.6.7.5 Meningoencephalitis

It is rare to isolate any of the Adenoviruses from either the cerebrospinal fluid (CSF) or the brain. However, several reports have directly detected adenoviruses in CSF (HADV3, HADV5, HADV6, HADV7, HADV7A, and HADV12) (Knipe et al., 2001). One patient with malignant lymphoma, immunosuppressed by chemotherapy, had an HADV32 isolated from brain at autopsy (Chou et al., 1973). There are other cases of meningoencephalitis in which viral isolation from extra neural sites or antibody titer increases have been used to make a diagnosis, especially associated with epidemic HADV7 pneumonia in children (Knipe et al., 2001). A case of sudden unilateral deafness was associated with an HADV3 infection of the nasopharynx (Knipe et al., 2001).

1.1.6.7.6 Gastrointestinal Diseases

The relationship between Adenoviruses and diarrhea has had a long and complicated history, but has been clarified considerably in recent years (Knipe et al., 2001).Because many Adenoviruses replicate efficiently in the intestine and are excreted in the stool, it was assumed that they would be strong candidates for causing diarrhea (Knipe et al., 2001). However, earlier epidemiologic studies generally found as many Adenovirus isolates in the stools of controls as in those with diarrhea (Ramos-Alvarez and Sabin, 1958). The failure to correlate Adenovirus growth from stool with clinical illness was a good example that Adenoviruses should not be designated as the cause of a whole spectrum of medical illnesses just because they can be cultured from the stool of an individual with a disease. Normal children can clearly shed Adenoviruses in stool and often

develop antibodies to the particular type grown (Hillis et al., 1973). These sub-clinical infections probably result in lifelong immunity. That young children with systemic adenovirus infections can develop diarrhea as an accompanying problem is well known but probably no more common than after any other systemic infection (Knipe et al., 2001).

1.1.6.8 Diagnosis

Antigen detection, polymerase chain reaction (PCR) assay, virus isolation, and serology can be used to identify adenovirus infections. Adenovirus typing is usually accomplished by hemagglutination-inhibition and/or neutralization with type-specific antisera. Since Adenovirus can be excreted for prolonged periods, the presence of virus does not necessarily mean it is associated with disease (Chmielewicz et al., 2005 b).

1.1.6.8.1 Serum neutralization

Type specific identification is classically performed by serum neutralization (SN) and /or hemagglutination inhibition (HI) assays, the type–specific determinants of HADVs recognized by SN are located primarily on the surface of the hexoncapsomere,but determinants are also present on the fiber and, to a lesser extent,on the penton proteins (Lu and Erdman, 2006).

1.1.6.8.2 Immunotyping

Immunotyping HADVs by classical methods is labor intensive, dependent on hyper immune polyclonal reagents that are in short supply, and results can be delayed for weeks. Moreover, interpreting assay results can be difficult, given extensive cross reactions between some Ad serotypes, particularly among species A and D viruses (Wigand, 1987). Type-specific monoclonal antibodies have been developed for a limited number of HADV, but these reagents are not widely available (Wood et al., 1997).

1.1.6.8.2 Direct Immunofluorescence (DFA)

Hereby, an Adenoviral antigen is detected using an antigen-specific antibody against it tagged with a fluorescent dye. This method is generally the first line of respiratory tract infections in nasopharyngeal aspirates, since it offers a straight-forward detection of viral antigen (D`Alessio et al., 1970).

1.1.6.8.3 Restriction fragment length polymorphism analysis

In the early 1980s, molecular typing of HADVs by genomic restriction fragment length polymorphism (RFLP) analysis was proposed as an alternative to immunotyping (Adrian et al., 1986). Genomic RFLP analysis offered a comprehensive assessment of genetic variation across the entire viral genome and remains a popular tool for strain characterization of some HADV serotypes. However, this method requires cultured virus, is labor intensive, and results for some serotypes can be difficult to interpret due to novel RFLPs (Lu and Erdman, 2006).

1.1.6.8.4 Polymerase chain reaction

Polymerase chain reaction (PCR) has grown in popularity as an alternative to immunotyping for identifying HADVs, offering a sensitive, rapid and readily accessible technology that can provide more definitive results (Chmielewicz et al., 2005 b). PCR assays using species- and type – specific primers targeting the hexon, fiber, and VA (The viralassociated RNA is a type of non-coding RNA found in Adenovirus RNA) coding regions have been described for some serotypes (Lu and Erdman, 2006). However, because the HVRs (hyper variable regions) comprise a large discontinuous region of the hexon gene, multiple PCR amplication and sequencing reactions are required. Moreover, HVR reference sequences have not been available for all HADV prototype strains, limiting the utility of this region for molecular typing (Lu and Erdma, 2006).

Recent studies have suggested that shorter regions of the hexon gene correlate with serotype and therefore may be more practical for routine molecular typing (Lu and Erdman, 2006). Sarantis et al. described a typing assay based on PCR and sequencing of HVR7 (Sarantis et al., 2004). Shimada et al. have proposed a similar assay that targeted a more conserved region of the hexon gene (Shimada et al., 2004). Quantitative PCR assays allow monitoring of the kinetics of the viral infection to distinguish between acute and persistent infections and estimation of the success of antiviral therapy (lion et al., 2003).

1.1.6.9 Treatment

Most infections are mild and require no therapy or only symptomatic treatment. Since there is no virus-specific therapy, serious Adenovirus illness can be managed only by treating symptoms and complications of the infection. Deaths are exceedingly rare but have been reported (Wu and Martinez, 1990).

1.1.6.10 Epidemiology of Adenovirus

Adenovirus infections occur worldwide in humans as well as in a variety of animals. With few exceptions, the human Adenovirus (HADV) serotypes are generally not pathogenic to animals, and animal Adenoviruses are only pathogenic within the species of origin (Taylor et al., 1977). Military recruits are highly susceptible to HADV associated ARD **(**Gray et al., 2000). In the absence of vaccine intervention,outbreaks are essentially continuous and result in large numbers of sick trainees, providing a unique setting to examine the molecular epidemiology and dynamics of transmission**.** Adenoviruses probably account for 3% of the infections in civilian populations (Cook et al., 1986). Serologic surveys have furnished some estimates of the prevalence of Adenovirus infections in various populations. Antibodies to HADV1, HADV2, and HADV5 are most common and are present in 40-60% of children (Brandt et al., 1969). The incidence of antibodies to HADV3, HADV4, and HADV7 is low at the same ages (Brandt et al., 1969). These antibody results probably explain why adults are uncommonly infected with HADV1, HADV2, and HADV5 but are more susceptible to infections with HADV3, HADV4, and HADV7 (Cook et al., 1986). During the surveillance for the virus watch studies, it was documented that only about 75% of the Adenovirus isolates were accompanied by an antibody response (Cook et al., 1986).

1.1.6.11 Transmission

The transmission of Adenovirus infection and disease varies from sporadic to epidemic. The pattern often correlates well with the viral serotype and the age (children or adults) of the susceptible population (Knipe et al., 2001). Fecal-oral transmission accounts for most infections in young children. Initial spread may occur by the respiratory route, but the prolonged carriage in the intestine makes the feces a more common source during both the acute illness and intermittent recurrences of shedding (Cook et al., 1986). The epidemiologic importance of the long latency in tonsil tissue is not known (Knipe et al., 2001).

1.1.6.12 The epidemic forms of Adenovirus

The epidemic forms of Adenovirus disease were studied in different ways from that of the sporadic endemic occurrences (Knipe et al., 2001). The epidemics of acute respiratory disease (ARD) were well known during World War II, and this awareness preceded the isolation and the characterization of the first Adenovirus by about one decade (Knipe et al., 2001). This ARD, which occurred almost exclusively in recently assembled military recruits, was most common in winter. It did not occur in seasoned personnel in close contact with the recruits and was later identified as an HADV4 or HADV7 infection in most outbreaks (Fredman and Engler, 1993). This disease rarely occurred in similarly congregated college students, suggesting that additional factors, such as more crowded sleeping conditions or the fatigue associated with basic training, contributed. In support of these cofactors has been the observation that ARD-causing Adenoviruses did not spread to civilian personnel in contact with the military (Knipe et al., 2001). In recruits congregated during the summer months, ARD often did not occur until the onset of colder weather in the fall. Influenza A could be distinguished because it affected both experienced and new recruits. Adenovirus-induced ARD often affected 80% of the recruits, with 20% to 40% hospitalized. The duration of infectivity was rather short; virus was not demonstrable after 4 days of illness (Knipe et al., 2001). ARD outbreaks were effectively controlled by vaccination of recruits in the first few days of military service; however, recent interruption in the supplies of Adenovirus vaccine has resulted in the reappearance of the epidemic form of ARD (Gaydos and Gaydos et al., 1995; Gray et al., 2000; Kolavic-Gray et al., 2002). Infection resulting in several of the Adenovirus syndromes can be acquired in hospitals and can be spread as nosocomial infections. Other adenovirus serotypes have probably been spread as nosocomial infections (Knipe et al., 2001).

1.1.6.13 Endemic Adenovirus Infections of Young Children

About 5% of ARD cases in children younger than 5 years of age are due to an adenovirus (Broor et al., 2007). The usual symptoms include nasal congestion, coryza, and cough. Other patients

may have an exudative tonsillitis that may be clinically indistinguishable from disease caused by the group A streptococcus (Harris et al., 1971). The respiratory symptoms are often accompanied by systemic manifestations, such as generalized malaise, fever, chills, myalgia, and headache (Knipe et al., 2001). The common serotypes are HADV1, HADV2, HADV5, and Ad6 and occasionally HADV3, which are endemic in most populations (Knipe et al., 2001). Sporadic cases may be indistinguishable from other viral respiratory infections, such as influenza, parainfluenza and respiratory syncytial virus (Bell et al., 1955). If conjunctivitis accompanies the signs and symptoms already described, the disease is designated as pharyngoconjunctival fever (Bell et al., 1955). The conjunctivitis is usually follicular, unilateral or bilateral, and characteristically mild. In more severe cases, pneumonia may occur. Adenoviruses serotype 14 is probably responsible for about 10% of the pneumonias of childhood (Louie et al., 2008). Most patients recover from these lower respiratory infections, but some epidemics of HADV7 have resulted in considerable mortality (Gray, 2006). Sequelae in those who recover may include bronchiectasis that can clinically manifest years after the primary infection.

1.2 Goals of this study:

1. To genotype the Adenovirus isolates from the 338 positive nasopharyngeal samples in babies' and children' aspirates collected at CBH between 2005 and 2010.

2. To assess the association between Adenovirus infections and patient age, place of residence,as well as the infection season.

3. To measure the clinical presentation of the patients when they were admitted to CBHwith Adenoviral disease cases (338 children).

1.3 Description of Caritas Baby Hospital:

Caritas Baby Hospital (CBH) is one of the main pediatric hospitals in the south of Palestine. It is located in Bethlehem and provides services mainly to patients from Bethlehem districts. Before Intifada started, babies from all over Palestine used to come for treatment at CBH.

Caritas Hospital consists of two main departments the inpatient wards and the outpatient clinics. The inpatient has a capacity of 80 beds including incubators. The inpatient division is divided into three wards. One ward is for premature babies and the other two wards are for babies and toddlers. The wards offer general pediatric medical treatments excluding surgery and intensive care. It has the necessary diagnostic laboratories and adjoining facilities, as radiology and physiotherapy department. The annual number of admissions range between 3000 to 3500.The outpatient clinic operates daily and evaluates on average 1600 patients annually.

1.4 Ethical consideration

This study was for scientific purposes only. It does not discriminate against any particular religion, or ethnic group. All the study information has been taken confidentially; patient's names and their private information were anonymous.

CBH ethical committee approved the participation in this research study (see 2.5; Appendix 1).

1.5 Validity and reliability

The laboratory of CBH is under direction of Dr. Musa Hindiyeh, a diagnostic clinical microbiologist, with double American board certification. First board is in Medical Technology from the American Society of Clinical Pathology (ASCP) and the second board is in Diagnostic Microbiology and Public Health (DABMM) from the American Society of Microbiology.

Other issues taken into consideration to increase the validity and reliability of results:

1. DFA (direct Immunofluorescence assay) results were read by technicians at CBH.
2. All reagents were within the expiration date.
3. Specimen transport media swabs were from COPAN, a well-known American company (www.copanusa.com)

2. Materials and methods

2.1 Study design

This study is case control study; cases were children diagnosed with Adenovirus (HADV) respiratory infection. The study controls were children within the same age group diagnosed with respiratory tract infections caused by Influenza A (Flu A) or Parainfluenza (HPIV) viruses.

2.2 Target population

The target populations for this study were Palestinian children from southern Palestine, particularly Hebron and Bethlehem districts, who were between few days and seven years old. The setting was classified as city, village or camp.

2.3 Sample setting

This is a cross sectional reterospective study; a non random purposive sample was used to collect data about all children with respiratory tract infections who were admitted to Caritas Baby Hospital. Further selection criteria for cases were:

1- Children between few days and seven years old
2- Children who were diagnosed with HADV respiratory tract infection.
3- Children residing in southern Palestine.
4- Children admitted to CBH with respiratory tract infection during all months of the year in the period between January 2005-December 2010
5- Severity and symptoms of disease at the time of admission to CBH.

In case of the control group, the selection criteria were:

1- Children between few days and seven years old
2- Children who were diagnosed with Flu A or HPIV respiratory tract infections.
3- Children residing in southern Palestine.
4- Children admitted to CBH with respiratory tract infection during all months of the year in the period between January 2005-December 2010.

2.4 The study setting

The study setting was carried out in southern Palestine districts of Hebron and Bethlehem, the setting was classified into city, village or camp. Figure 2.1 indicates the area, where the children reside.

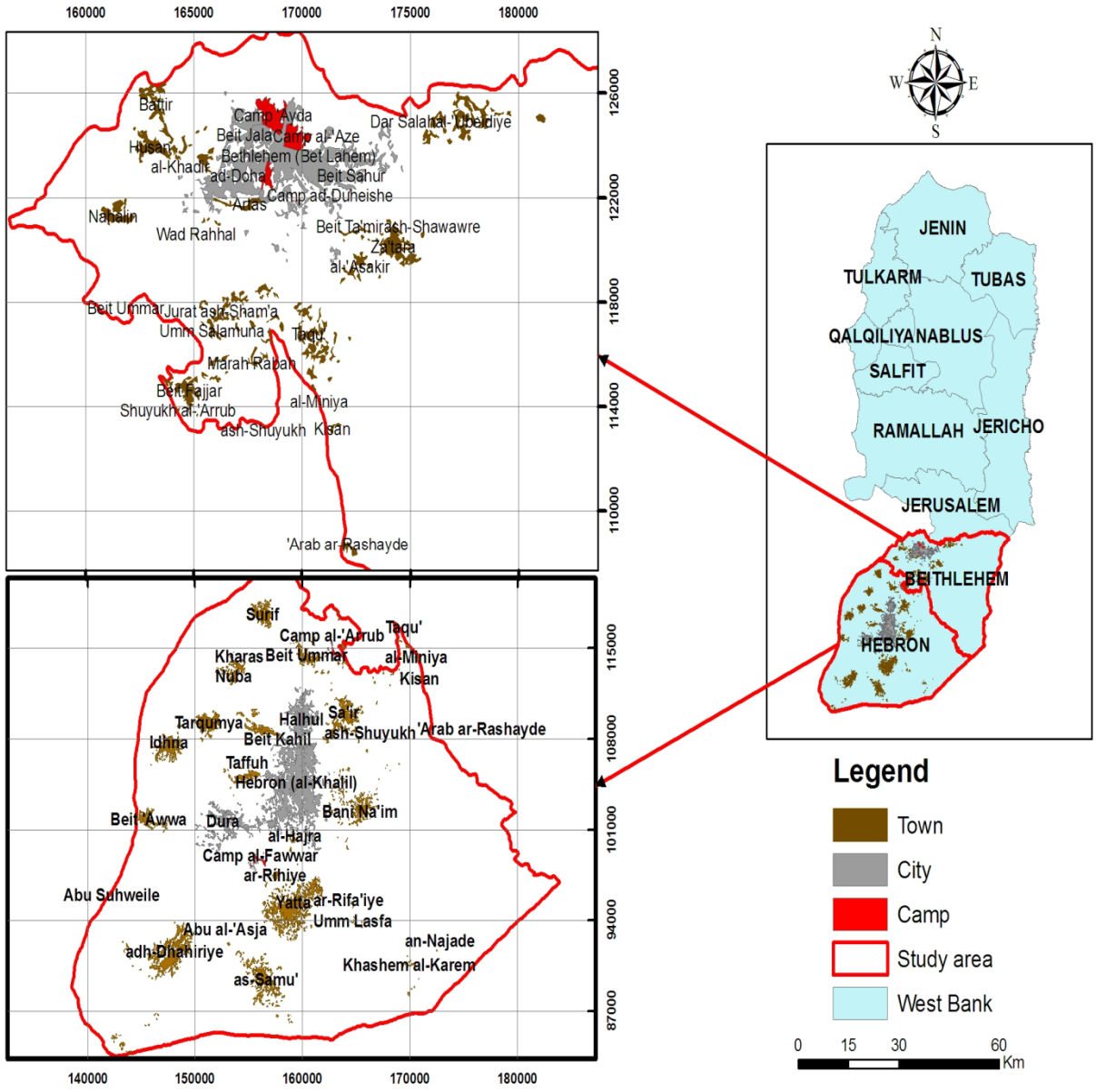

Figure 2.1: Residency of children admitted to the CBH with respiratory tract infection caused by Adenovirus. The figure was generated by the environmental research laboratory-AL-Quds University.

2.5 Ethical approval

This study required both medical records of the children with Adenoviral infection as well as clinical samples on which the analysis were to be performed.

for this regard, an application with three main requests was written and submitted to the ethical committee of the CBH. The application was approved (Appendix 1).

A. First request was directed to Dr. Musa Hindiyeh, director of CBH laboratory in order to obtain approval for checking the general lab records of children with confirmed respiratory tract infections. After approval, out of a list with over 8000 records, children with diagnosed HADV, FLU A and HPIV infections were selected. The general records regarding age, sex, Admission date and residency were collected.
B. Second request was also directed to Dr. Musa Hindiyeh to obtain the Nasopharyngeal aspirates (NPAs) frozen samples of the children selected from the lab records with HADV respiratory tract infection.
C. The third and final request was directed to the director of the CBH to obtain permission to look into the medical files. This approval was necessary to obtain the clinical data, i.e. symptoms on admission and other clinical features of the children with HADV infection.

2.6 Medical records

2.6.1 General records

The general records of the respiratory tract infection cases, which included age, sex, residency and admission date, were collected from the laboratory data of the CBH upon ethical approval A (see above). These records were verified to decide which samples met the inclusion criteria for the study. There were 343 cases of HADV infection, 466 of HPIV and 291 of Flu A between the Jan 2005 and Dec 2010. Five of HADV cases, 15 of HPIV and 13 of Flu A were excluded due

to the residency in northern part of Palestine. 338 cases of HADV infection, 451 of HPIV and 278 of Flu A were included in this study.

2.6.2 Medical background records

Upon ethical approval (see 2.5; Appendix 1), data from 150 files of Adenovirus infection cases were collected. These data included the clinical picture of children on admission and intake of antibiotics. The data were analyzed using SPSS to conclude the relevance between the clinical variables and the patients' cases.

2.7 Data collection and initial analysis

All data were collected in excel sheets, percentages and charts were created directly through these excel sheets. For this data were arranged in an excel file, where each case, residency, age, sex, admission date (day, month and year) and type of infection (HADV, HPIV or Flu A) were included. For Adenovirus infection cases, the clinical presentation infection and the resulting serotypes were added.

2.8 SPSS data analysis

All data included in this study were analyzed utilizing the SPSS program. The SPSS analysis were performed based on the excel data sheet records from 2.7 (see above).

2.9 Specimen collection

Nasopharyngeal aspirates (NPAs) samples were collected from the respiratory tract of each patient at CBH according to rules and ethical consideration at CBH. The collection of NPAs demanded a trained nurse and the technical procedure for collection was described in Abu Diabet

al (Abu Diab et al., 2008). The DFA detection of Adenovirus in NPA specimen was also described in Abu Diab et al (Abu Diab et al., 2008). For this, NPAs from each patient were sent to the CBH laboratory at room temperature within 30 min of specimen collection. The NPAs were vortexed for 30 s and centrifuged at 300 x *g* for 10 min. The cell pellets were washed three times with PBS washing buffer (phosphate buffer saline), and the cells were re-suspended in 0.5 ml saline before a ~20µl cell suspension was spotted in the wells of an acetone-cleaned glass slide. The slides were air dried before they were fixed in cold acetone for 10 min. The cell concentrations were adjusted in NPA samples with large amount of secretions. A Light Diagnostics respiratory direct fluorescent antibody assay (DFA) viral screening and identification kit (Chemicon International; now part of Millipore) was used to stain for adenovirus, Influenza A and Parainfluenza viruses. As recommended by the manufacturer. Fluorescein isothiocyanate-labeled antibodies against each virus were added to the appropriate wells; and the slides were incubated at 37°C for 30 min in a humid chamber. The slide was then washed with 0.5% Tween in PBS for 45 s and then with distilled water for 15 s. Finally the slides were air dried and the epithelial cells spotted on each slide were evaluated under a Hund H 600 fluorescent microscope at x10 magnification. Specimens were reported as inadequate if the number of epithelial cells was less than 20 cells per 10X field and no positive cells were spotted (Landry et al., 2000). In order to evaluate the effectiveness of specimen collection, the epithelial cells were enumerated semi quantitative at 40X magnification according to the following criteria: +1, 1 to 10 cells; + 2, 10 to 20 cells; and +3, >20 cells. All slides were screened for positive fluorescing cells at x10 magnification, and the results were confirmed at 40X magnification. Positive cells were also enumerated semi quantitative at 40X magnification according to the following criteria: +1, 0 to 1 cell; + 2, 1 to 10 cells; + 3, 10 to 20 cells; and + 4, >20 cells (Abu Diab et al., 2008).

2.10 Extraction of HADV DNA from Nasopharyngeal aspirates samples

Frozen NPAs at the CBH from 2005-2010 were transported on ice to the Virology laboratory; they were either frozen or subjected directly to DNA extraction.

For Adenoviral DNA extraction, Qiagen DNA extraction kit (Qiagen.cat no.51304) was used. The extraction steps were performed according to the manufacturer's instruction as follows:

1. 20 µlQiagen Proteinase K were pipetted into the bottom of a 1.5 ml microcentrifuge tube.
2. 200 µl samples were added to the microcentrifuge tube. If the sample volume is less than 200 µl, PBS was added to adjust the amount.
3. 200 µl Buffer AL were added to the sample, mixed by pulse-vortexing for 15 s.
4. Mixture was incubated at 56ºC for 10 min followed by a short spin down
5. 200 µl ethanol (96-100 %) were added to the sample, mixed by vortexing and briefly centrifuged to remove drops from the inside of the tube lid.
6. Mixture from step 5 was applied carefully to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and column in the collection tube was centrifuged at 8000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2ml collection tube, and discard the tube containing the filtrate.
7. 500 µl Buffer AW1 were added to the QIAamp Mini spin column and add without wetting the rim and centrifuged at 8000 rpm for 1 min. Filtrate was discarded.
8. The QIAamp Mini spin column was replaced in a clean 2 ml collection tube, 500 µl Buffer AW2 were added and. Filtrate was discarded and centrifuging step was repeated.
9. Finally, the QIAamp Mini spin column was placed in a clean microcentrifuge tube, 200 µl elution buffers was added to the column and centrifuged at 14000 rpm for 3 min. The filtrate in this step resembles the isolated DNA.

2.11 Genetic detection of Adenoviral Hexon gene

2.11.1 Design of PCR primers specific to adenovirusHexon gene:

Initially a primer pair to amplify a hexon gene fragment of 800bp was designed. However, these primer pairs resulted in low frequency of amplification or no amplification at all due to the high genetic variety of the Hexon gene. Therefore, well adjusted primer pairs in nested PCR mode were utilized (Lu and Erdman, 2006, table 2.1). These Adhex primer pairs were designed to bind to all yet known 53 serotypes by inducing many variable bases to cover different point mutations leading to the different serotypes and Inosin (Lu and Erdman 2006, table 2.1). Inosine is a purine (which occurs naturally in tRNAs) that can form base pairs with cytidine, thymidine, and adenosine. Inosine is used in primers at positions where any of the four bases might be required due to sequence heterogeneity as is the case with HADV.

Primer (location on gene)	Primer sequences	Source
AdhexF1 (298-323)	5-txctttgacatxcgxggxgtxctxga-3	Erdman; 2006
AdhexR1 (1157-1136)	5-ctgtcxacxgcctgrttccaca-3	Erdman; 2006
AdhexF2 (328-350)	5-ggyccyagyttyaarccctaytc-3	Erdman; 2006
AdhexR2 (1122-1097)	5-ggttctgtcxcccagagartcxagca-3	Erdman; 2006

Table 2.1: Primers used to amplify Hexon gene of Adenovirus. I= Inosin (A, T, C or G), X= (G, A, T or C), R= (A or G), Y= (C or T), according to IUPAC nucleotide code.

2.11.2 Primer location

To determine the site of the selected primer (see table 2.1), the hexon gene of different HADV genotypes were downloaded from the Pub med website http://www.ncbi.nlm.nih.gov/pubmed/ to the Clone Manager Suite 7 program (scientific and educational software, www.scied.com). The primer sequences were searched using the search machinery of the program. The 5' and 3' of each primer was determined on the gene. The distance between the 5' of the external forward primer (AdhexF1) and the 5' of the external reverse primer (AdhexR1) resemble the size of the

PCR product to be expected after the nested PCR reaction with the two primer pair (the external and the internal primer pairs).

2.11.3 PCR conditions

A total PCR reaction of 25 µl included 8.5 µl extracted DNA (see 2.9), 1µl of each primer; AdhexF1, AdhexR1, AdhexF2 and AdhexR2 (each 10 pmol/µl) and 12.5 µl PCR master mix (Ready Mix PCR Master Mix of Thermo scientific, Cat. No. AB-0575\Dc\LD). The reaction was carried out in PCR tube (0.2 ml Axygen INC, USA) using Swift™ MaxPro Thermal Cyclers (ESCO Global, USA). The PCR reaction started with a single hot start step for 5 min at 95ºC followed by 35 cycles in the following order, 1 min at 95ºC for denaturation, 1 min at 45 ºC for annealing and 2min at 72 ºC for extension respectively. A following additional extension step was performed for a further 10 min at 72 ºC, assuring the complete extension of the amplified product. The reaction was then cooled down to 4ºC and either frozen at -20ºC or directly separated as in 2.10.4.

2.11.4 Detection of PCR product

To detect the amplified gene product of the PCR reactions, agarose gel electrophoresis was used to separate the PCR product of expected 794 bp. 1 % agarose (Amresco) gel was prepared in 1x TAE (10 x TAE = 400nM Tris-HCL, pH 8.3; 200mM Na-Acetat; 20 mM EDTA). The agarose was boiled until it was well dissolved, ethidium bromide or SYBER green (Invitrogen, cat number. S33102, safe DNA gel stain) was added when agarose suspension cooled down to 40ºC, carefully mixed, poured into agarose gel casting system (BioRad, UK or Cleaver**,** U.S.A) and a desired comb was inserted. 8 µl of the PCR product was added directly into the gel well along with the DNA size control; 2 µl from 100 bp marker (gene ruler express DNA ladder,

(Fermentas, cat number. SM1558) added to detremine the correct band size. Finally, the migrated bands in the agarose gel (100 mv for 30min using Bio Rad power supply) were visualized under UV light. A digital image of the gel was taken using digital camera of the gel documentation system (Pharmacia Biotech).

2.11.5 PCR Purification

To purify the positive PCR product, a MinElute PCR purification Kit (Qiagen) was employed. This step is essential to get rid of PCR components and concentrate the desired PCR product for sequencing purposes or further analysis. Purification kit (Cat. No. 28004) was used, as follows:

1. 5 volume of Buffer PB was added to 1 volume of the PCR reaction mix. In our case, 100 µl of buffer PB were added to 20 µl PCR reaction. The color of the mixture is expected to turn yellow; otherwise Sodium acetate should be added.

2. To bind DNA, the sample mixture (from step 1.) was applied to the MinElute column and centrifuged at 17,900 g for 1 min.

3. The flow-through was discarded and 350 µl Buffer PE (wash buffer) was added to the MinElute column and centrifuged for 1 min at maximum speed (17,900 g).

4. Flow-through was discarded and centrifugation step repeated.

5. For elution of the bound DNA, the MinElute column was placed in a clean 1.5 ml microcentrifuge tube. 10 µl Elution Buffer (10 mMTrisHCl, pH 8.5) or water was added to the center of the column's membrane. After 1 min standing at RT, the column/microcentrifuge tube was centrifuged for 1 min at 17,900 g.

6. 3 µl purified DNA was analyzed on a gel to verify the amount of eluted DNA. The result was photographed using digital camera of the gel documentation system (Pharmacia Biotech). This

photo reflects the DNA quality and quantity and was sent to the Bethlehem Heredity center along with the remaining 7 µl and the forward primer AdhexF2 for sequencing.

2.12 Sequence analysis of hexon gene

Sequencing was performed at the heredity lab of the Bethlehem University using sequencer machine ABI PRISM 3130 Genetic Analyzer. The sequencing PCR reaction was performed with the reverse or forward primer in each reaction and BigDye® Terminator v1.1 Cycle Sequencing Kit(Applied Biosystems, USA, cat no. 4337451-100). Once the sequence was ready, we received an email with the sequences attached.

2.12.1 General overview of sequencing results

The hexon gene sequences were readable using the Chromas lite program, Technelysium Ltd (www.technelysium.com.au/chromas.html). Using the NCBI blast machine (http://blast.ncbi.nlm.nih.gov/Blast.cgi), sequences were blasted for comparison with other known published adenoviral serotypes. The adenoviral genotypes with the highest maximal identification with the Palestinian samples' sequences were documented for initial orientation of available Palestinian serotypes. The accurate sequence analysis was performed using the DNASTAR program (see below).

2.12.2 Reference sequences

In order to identify the adenoviral sequences revealed from Palestinian samples, published complete hexon gene serotypes were used as a reference. Using the MegAlign program searching machinery (Lasergene version 8, DNASTAR Inc., Madison, WI, USA), complete hexon genes were downloaded and saved. For precise and overall detailed alignment, serotypes of all subgenus (A-F) were downloaded beside the serotypes and subgenus found to be aligning with the Palestinian samples in the initial sequence analysis (see 2.12.1). The published reference sequences were saved and referred to by the name of the serotypes and its accession number in brackets as published by the ncbi (www.ncbi.nlm.nih.gov). The Palestinian samples' sequences hold the number given to the sample during this work and the year of sampling. The samples of different years were given different colors to allow fast overview of the different years.

2.12.3 Accurate sequence analysis of Palestinian human adenovirus

2.12.3.1 Sequence alignment

An accurate analysis of the Palestinian sequences was accomplished using the MegAlign program (Lasergene version 8, DNASTAR Inc., Madison, WI, USA). All alignments were made using the Clustal W method and the following alignment conditions:

Pairwise alignment was always slow accurate with gap penalty 10; gap length 0, 10 and DNA weight matrix IUB. As for the multiple alignment gap penalty was also 10, gap length 0.20. These parameters were suggested by the program manager to fulfill the most ideal sequence alignment. Hereby clustal W method aligns sequences using the method of Thompson et al. (1994). Clustal W method was designed to create more accurate alignments than clustal V when alignments include highly diverged sequences. Gap penalty is the amount deduced from the alignment score for each gap in the alignment. Gaps of the different sizes carry the same penalty, gap length penalty is the value deducted from the alignment score after first multiplying it by the

length of gaps. Longer gaps have a greater penalty than shorter gaps. For example of alignment, please see figure 3.11.

2.12.3.2 Building the phylogenetic tree

Palestinian hexon gene and reference hexon gene sequences were downloadedinto the MegAlign program for DNA analysis (DNASTAR Inc., Madison, WI, USA). An initial DNA based phylogenetic analysis was generated to gain an overview of all occurring Palestinian serotypes from all sequenced samples. In this phylogenetic analysis case, the complete Palestinian sequences were aligned with the complete reference hexon genes of HADV-1, HADV-2, HADV-3 and HADV-5. This phylogenetic analysis is not precise, but it gives a good orientation of the occurring Palestinian Adenovirus serotypes overall.

The accurate phylogenetic analysis however, was based on the amino acid sequences of the variable region (HVR1-6) of the hexon protein (position 131 to 331 as defined by Crawford-Miksza and Schnurr, 1996) and emphasized by Lu and Erdman (Lu and Erdman, 2006). For this the reference HADV serotypes (2.12.2) were aligned from amino acid 131 to 331. Each Palestinian sample's DNA sequence was first only aligned with its homology serotype DNA sequence (of the region amino acid 131-331). The sequence of the Palestinian sample was compared with the reference homology serotype in the MegAlign and the Chromas program. This way, eye inspection was used to emphasize the nucleotide mismatches and gaps shown by the MegAlign and the sequence reading of the Chromas program. The Palestinian sample sequence of the amino acid region 131-331 was saved for the yearly alignment. Not all Palestinian sequences were used in this alignment, but the most convincing clear sequences were preferred to allow a precise genetic analysis.

The most accurate Palestinian sequences (region 131-331) of each year were finally aligned with the amino acid sequences of the reference hexon genes (region 131-331) and subjected to

phylogenetic tree analysis. The phylogenetic tree was generated by using the neighbor-joining method (MegAlign-DNASTAR program), built based on the percent identity and divergence of all sequences to each other as calculated by the MegAlign program. The confirmation of this analysis was ensured by adding the bootstrapping value to the phylogenetic tree as calculated by the MegAlign program.

The Bootstrap value is a popular way of evaluating the reliability of an inferred phylogenetic tree. The first step in a bootstrap analysis is to re-sample the alignment columns with replacement, i.e., in the re-sampled alignment, a given column in the original alignment may occur two or more times, while some columns may not be represented in the new alignment at all. The re-sampled alignment represents an estimate of how a different set of sequences from the same genes and the same species may have evolved on the same tree. For this a number of trials and a random seed number are essential. Hereby the number of trials option indicates the number of times to compare the displayed tree with random tree constructs that use the neighbor-joining methodology. The random seed option makes bootstrapping consistent with the Clustal interface. The number of trials used in this analysis was 1000, while the random seed was 111 as suggested to be the optimal range by the program maker of the MegAlign to fulfill the optimal analysis.

3. Results

3.1 Respiratory tracts infections cases in caritas baby hospital:

Between Jan 2005 and Dec 2010, over 8000 cases of children with respiratory tract infections were admitted to the Caritas baby hospital in Bethlehem. 14% of these cases were respiratory tract infections caused by Adenovirus (HADV), Influenza A (Flu A) and Parainfluenza (HPIV). There were 338 cases of HADV, 278 of Flu A and 451 of HPIV respectively. The children were showing different disease severity of the upper respiratory tract infections, they were different in age, place of residency and other parameters.

3.1.1 Residency of infected children

Most children admitted to the CBH are generally from southern Palestine; Hebron and Bethlehem districts and so was the case for the children samples included in this study. The few cases from Jerusalem and northern Palestine were excluded from the study. Children were residing in the cities, villages or the camps in both districts. Interestingly, the highest infection rate for either virus in our study sample was in children residing villages.

68% of Adenovirus cases were from villages, while 21% were residing refugee camps and 11% came from cities. In case of Parainfluenza infection, 72% were from villages, 19% from refugee camps and 9% from cities. Finally 75% of Influenza cases were from villages, 15% from refugee camps and 10% from cities (figure 3.1).

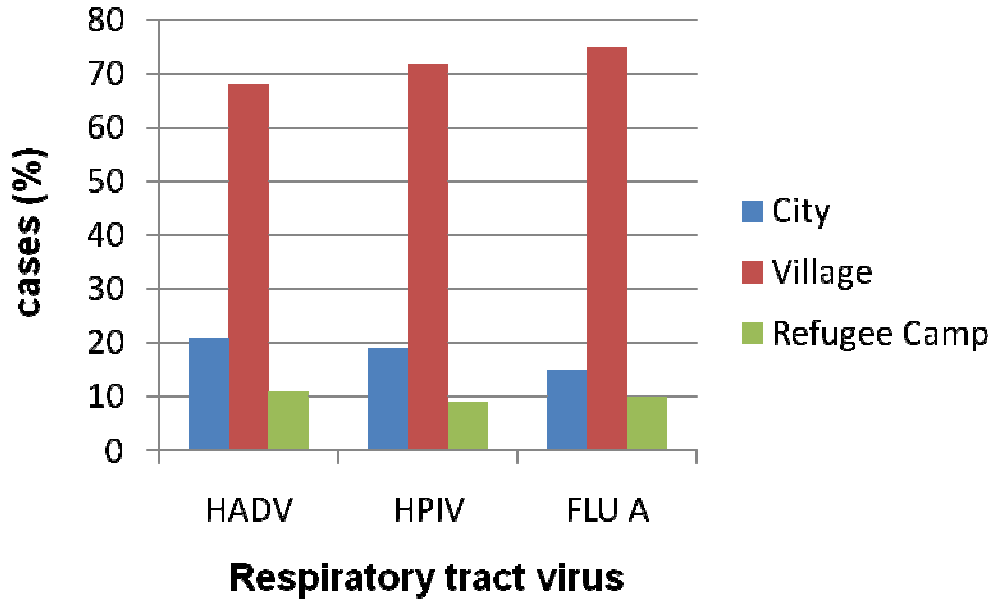

Figure 3.1: Residency of children admitted to CBH between January 2005 and December 2010 with either HADV, Flu A or HPIV. The percentage of cases was calculated from excel sheet showing data of each single case.

3.1.2 Age of infection

The children infected with either viruses; HADV, Flu A or HPIV were from the same age group categorized in this study. In our study sample, children who were few days old until one year of age (0-1) showed the highest rate of infection for all three viruses; 62% for HADV, 73.7% for HPIV and 66% for Flu A (figures 3.2). The infection rate decreased rapidly in children older than one year (1.1-2) represented by 27% cases for HADV, 16% for HPIV and 15% for Flu A (figures 3.2).

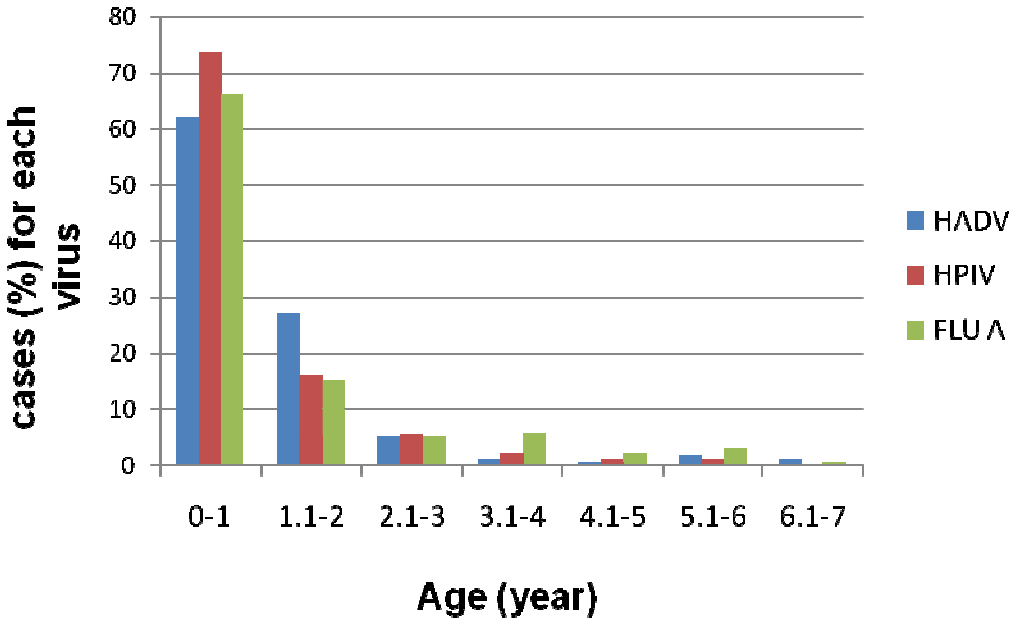

Figure 3.2: The age of children admitted to CBH between Jan 2005 and Dec 2010 with either HADV, Flu A or HPIV. The percentage of cases was calculated from detailed excel sheet showing data of each single case.

3.1.3 Sex of infected children

In our study sample 60% of infection cases with HADV, HPIV and FLU A were males compared to 40% females (figure 3.3).

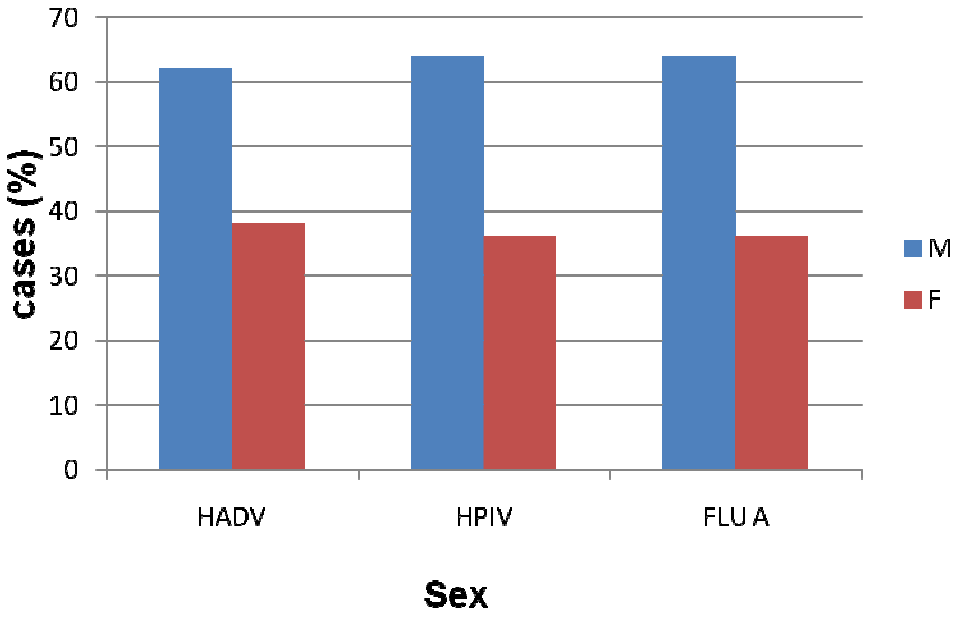

Figure 3.3: The percentage of male-Female among children admitted to CBH between January 2005 and December 2010 with respiratory tract infections caused by HADV, HPIV and FLU A respectively.

3.1.4 Infections per year

The infection rate per year was different for each virus. Adenoviral infections were increasing every year; while only 40 cases were detected in 2005, there were almost 100 ceases in 2010 (figure 3.4). In case of Parainfluenza, the infection rate started to increase in the year 2007 and was stable for the last three years. Most interesting was the infection rate of the Influenza A virus, which showed no clear tendency of increasing or decreasing until the last two years (figure 3.4). Most Flu A cases were detected in the year 2009, while no one single case was detected in 2010 (figure 3.4).

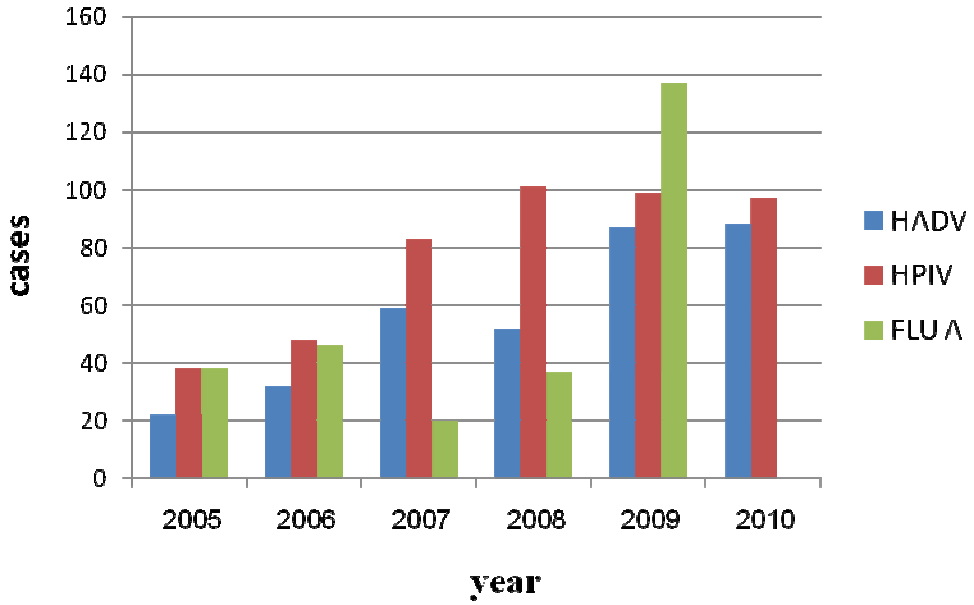

Figure 3.4: Yearly infection rate of HADV, HPIV and FLU A admitted to CBH between January 2005 and December 2010. The actual number of cases is shown on the y axis.

3.1.5 Season of infection

While age, residency and sex were similar for the three respiratory tract viral infections, the season of the respiratory tract infections clearly varied. There was no season of occurrence in case of Adenovirus or Parainfluenza infections, while Influenza occurred mainly in winter season (figures 3.5 A). Adenovirus infections occurred all over the year with an elevation of cases detectable in the months of March to May (figure 3.5 B). Interestingly, in the year 2010, when the highest number of Adenoviral cases was detected, the highest tendency of occurrence shifted to January (figure 3.5 B). On the other hand, the number of summed Parainfluenza cases for all the years was highest in May, but no clear tendency of infection was detectable (figure 3.5 A and C). The overall infections of HADV and HPIV varied every year, while Flu A clearly occurred between November and January every year (figures 3.5 B-D).

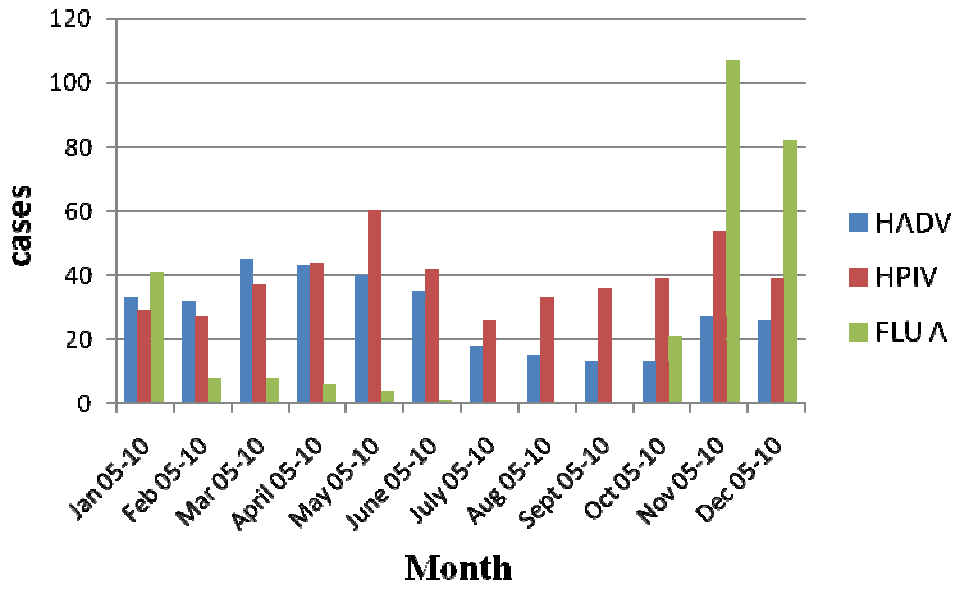

Figure 3.5A: Comparison of HADV, HPIV and Flu A monthly cases between January 2005 and December 2010. The actual number of is given on the y axis.

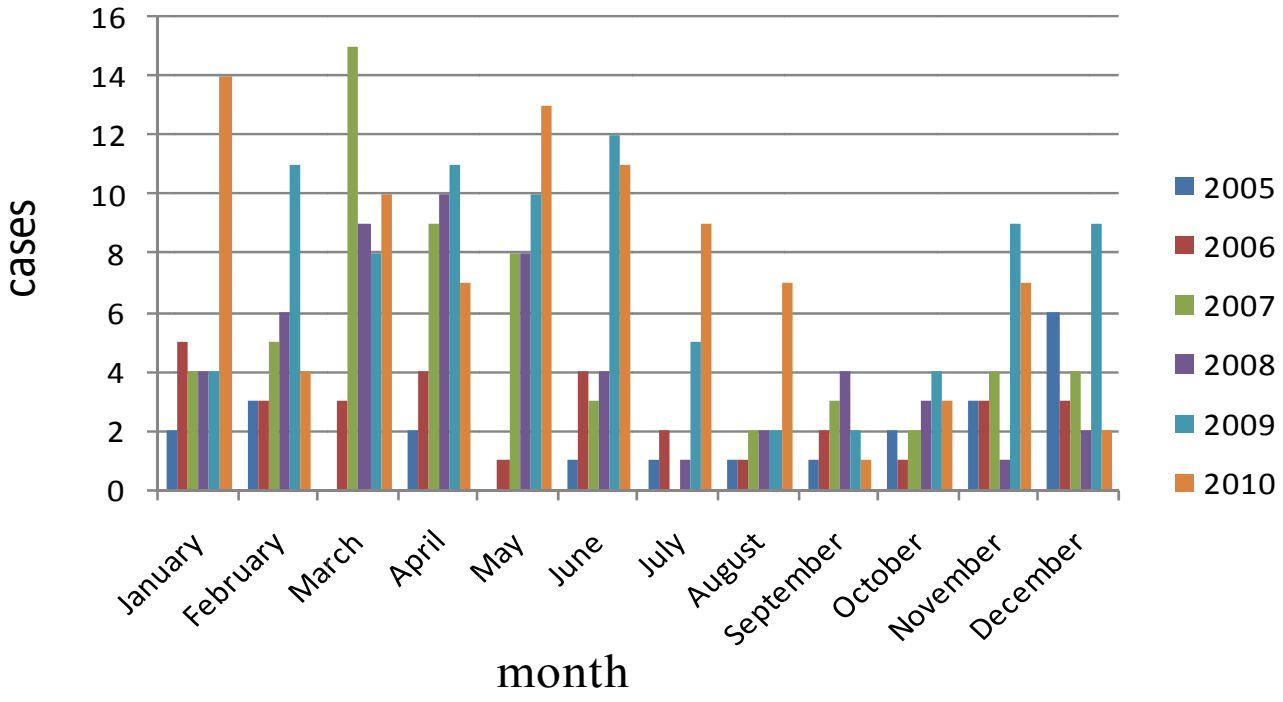

Figure 3.5 B: HADV cases per month from January 2005 to December 2010. The actual number of cases each month and for each year was used for this analysis.

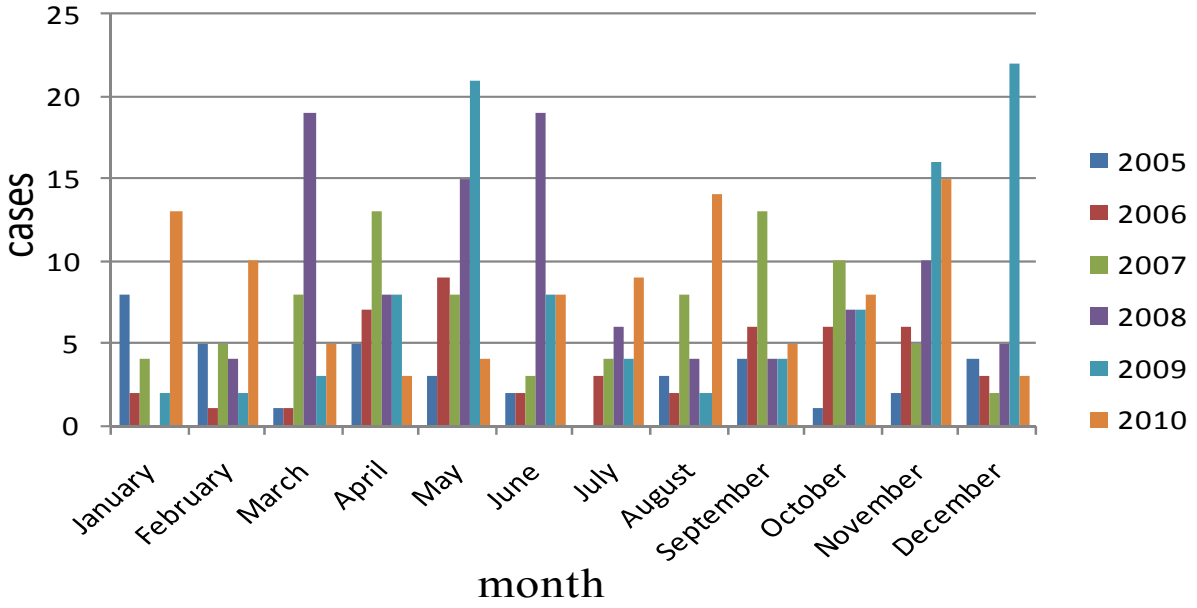

Figure 3.5 C: Parainfluenza infection per month from January 2005 to December 2010. The actual number of cases each month and for each year was used for this analysis.

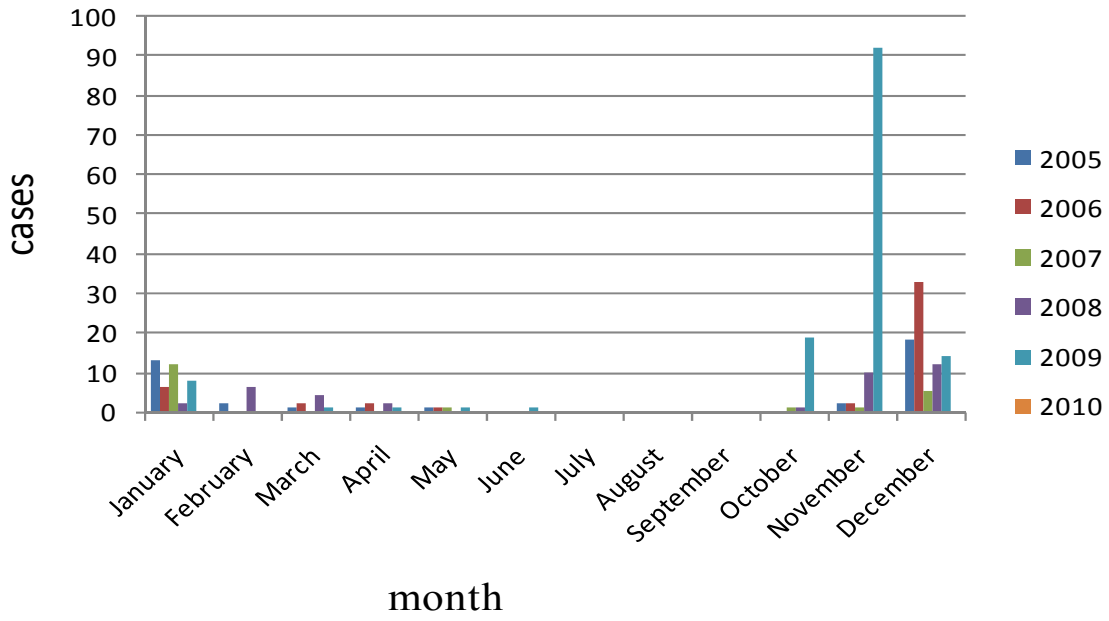

Figure 3.5 D: Influenza A infection per month from January 2005 to December 2010. The actual number of cases each month and for each year was used for this analysis.

3.1.6 Clinical features of Adenovirus infected children

To check the severity of adenovirus infections, medications and other clinical features for the Adenoviral respiratory tract infections included in this study, a sub sample (150 cases) was chosen randomly from the total of 338 cases. The medical files of these cases were studied and the results were summarized (see below).

3.1.6.1 Clinical presentation (severity)

The medical charts of the children showed clearly that Bronchitis, Bronchopneumonia (33.3%) and upper respiratory tract infections (URTI, 24.7%) were the main diagnosis of admission (figure 3.6). In 2-5% of the cases, children suffered also from sepsis, Gastroenteritis, Otitis media, Meningitis, breathing difficulty, dehydration, Tonsillitis, congenital heart disease (CHD), conjunctivitis, chronic renal failure, yellow skin and febril convulsion (figure 3.6). Single cases of the children suffered from croup, skin rash, hypoglycemia, anemia and cystic leukomalacia (referred to as others; figure 3.6).

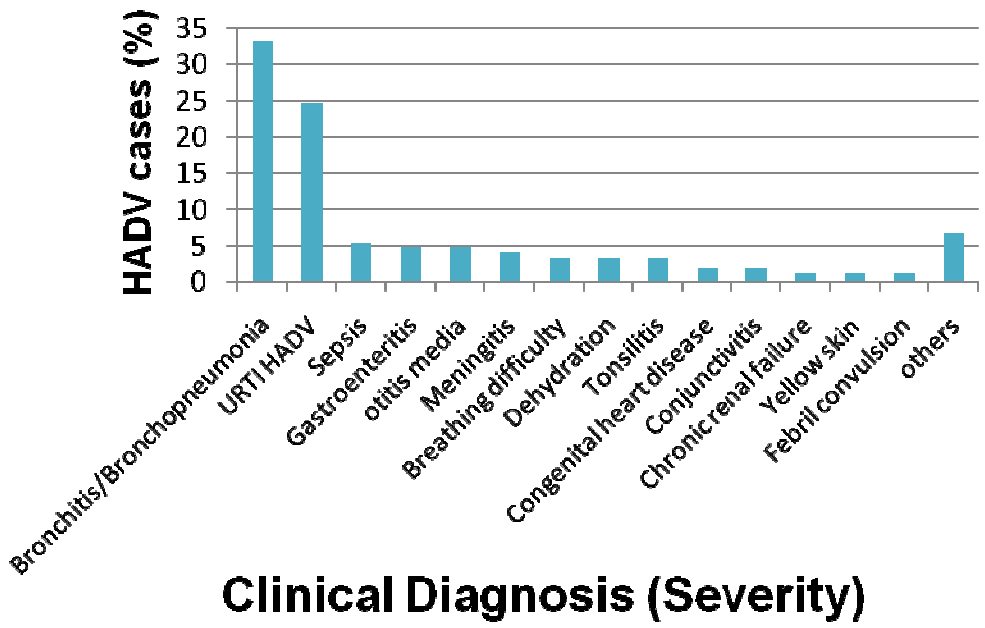

Figure 3.6: Clinical diagnosis of HADV sub sample of cases admitted to the CBH between 2005 and 2010. The cases with each symptom were summed and the percentage was calculated in relevance to the total number of HADV cases (338).

3.1.6.2 Antibiotic intake

In our subsample most children who were admitted to CBH and found to have respiratory tract infection have taken Antibiotics as clearly stated in the medical files (70%). Only 30% of the children were not subjected to antibiotic treatment (figure 3.7).

3.1.6.3 Fever and cough:

Children admitted to the CBH with adenoviral infection suffered from fever and cough (figure 3.7). While most children suffered from fever 80%, only 50% of them had cough symptoms (figure 3.7).

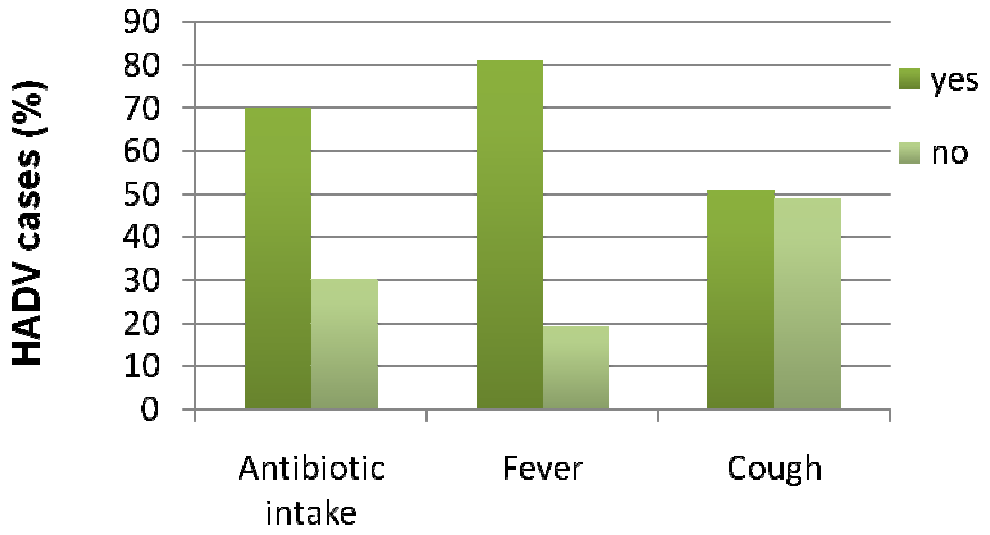

Figure 3.7: Antibiotic intake, fever and cough symptoms of HADV sub sample cases. The percentage of cases for each symptom was calculated in relevance to the total number of sub sample cases.

3.2 Genetic analysis of the Adenoviruses circulating in southern Palestine

3.2.1 Hexon gene PCR product

All 338 NPA samples were subjected to DNA extraction followed by amplification reaction of the HADV Hexon gene. The amplified PCR products were all around the expected Hexon gene fragment of 794 bp. Interestingly, many samples' amplification products (PCR product) varied in size showing either smaller or bigger fragments around the 794 bp (Figure 3.8 A and B). These variations were detectable all over the years and had a maximum plus or minus of 50 bp as compared to the DNA marker. On the other hand, a slight DNA smear was detected beside the clear PCR fragment. This smear is known to PCR products amplified using insosine rich primer pairs as this was the case here.

Figure 3.8 represents one of the amplification results of the year 2005. This picture was consistent all over the years. Most samples showed a very clear amplification band, while two samples (lane 10 and 11, figure 3.8 A) showed either very week amplification or no amplification at all. This was explained by the fact that these samples were either week positive in the DFA or that the DNA extraction was not successful. Interestingly, all samples' amplifications of the year 2010 were very successful, which is probably due to the fact that the samples were pretty fresh if compared with the samples of older years.

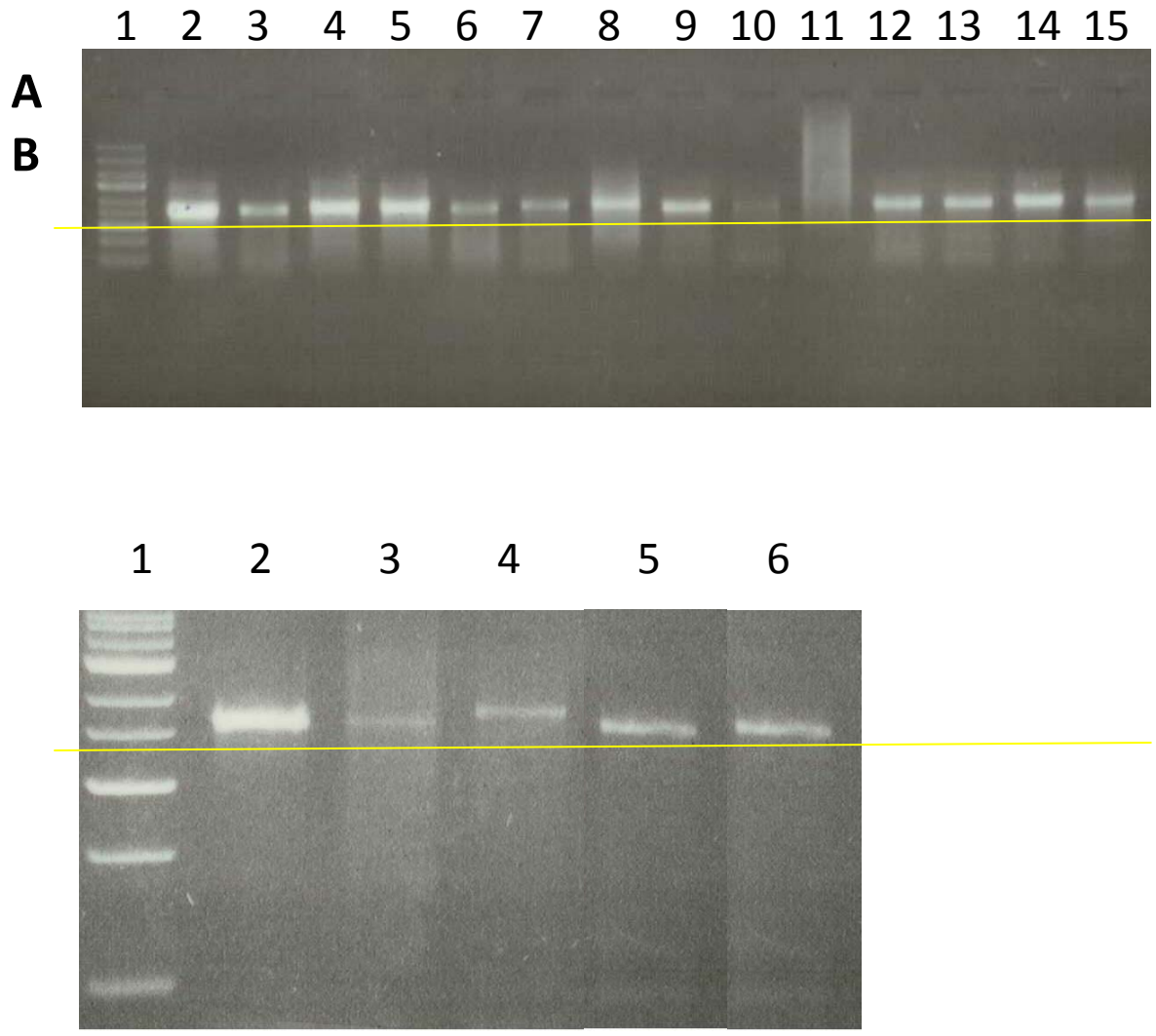

Figure 3.8.**A**: An example for HADV hexon gene amplification product from samples of the year 2005. Lane 1: marker; lane 2: 1-2005; lane 3: 2-2005; lane 4: 3-2005, lane 5: 4-2005; lane 6: 5-2005; lane 7: 6-2005; lane 8: 7-2005; lane 9: 8-2005; lane 10: 9-2005; lane 11: 10-2005; lane 12: 11-2005; lane 13: 14-2005; lane 14: 13-2005; lane 15: 15-2005. Please see also 2.11.4.
B: An example of HADV hexon gene product amplification elution from samples of the year 2005. Lane 1: marker; lane 2: 3-2005; lane 3: 5-2005; lane 4: 7-2005; lane 5: 13-2005; lane 6: 14-2005. For experiment details please see also 2.11.4 and 2.11.5.

3.2.2 Sequencing of the Adenoviral strains occurrence

Thirteen percent (44 samples) of HADV cases from all years were subjected to the sequencing analysis. Sequencing of 10% of samples for genetic analysis is scientifically very representative percentage to give an overall idea about genetic sequence of a species in a geographical area. The sequencing results showed clearly, that all the amplified fragments belonged to the Hexon gene of HADV as identified initially using the blast machinery of the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 44 samples sequenced were chosen randomly taking the different sizes of PCR product (see 3.2.1) and its quality into consideration.

3.2.3 Adenovirus serotypes in Palestine

3.2.3.1 General sequence analysis

Already the initial blast of the received sequences revealed that different HADV serotypes were represented in the Palestinian children. However, some HADV serotypes were more dominant in the Palestinian children. HADV serotype 2 (HADV-2); human adenovirus group C and HADV serotype 3 (HADV-3); human adenovirus group B were represented in 31.82% and 45.45% of the samples respectively (figure 3.9). Less dominant were another serotypes of the human adenovirus group C; HADV serotype 1 (HADV-1) and HADV serotype 5 (HADV-5), which were represented in 15.9% and 6.82% of the samples respectively (figure 3.9).

Interestingly, in 5 Palestinian cases of HADV-1, the DNA sequences also aligned 80% with other human adenovirus group C types; HADV-6 and the very recently described HADV-57 (Seto et al., 2010).

Taken together, these results show that the most dominant subgenus of human adenovirus in southern Palestine is group C, represented by serotypes HADV-1, HADV-2, HADV-5, HADV-6 and HADV-57. The second dominant human adenovirus subgenus is B, represented only by serotype HADV-3.

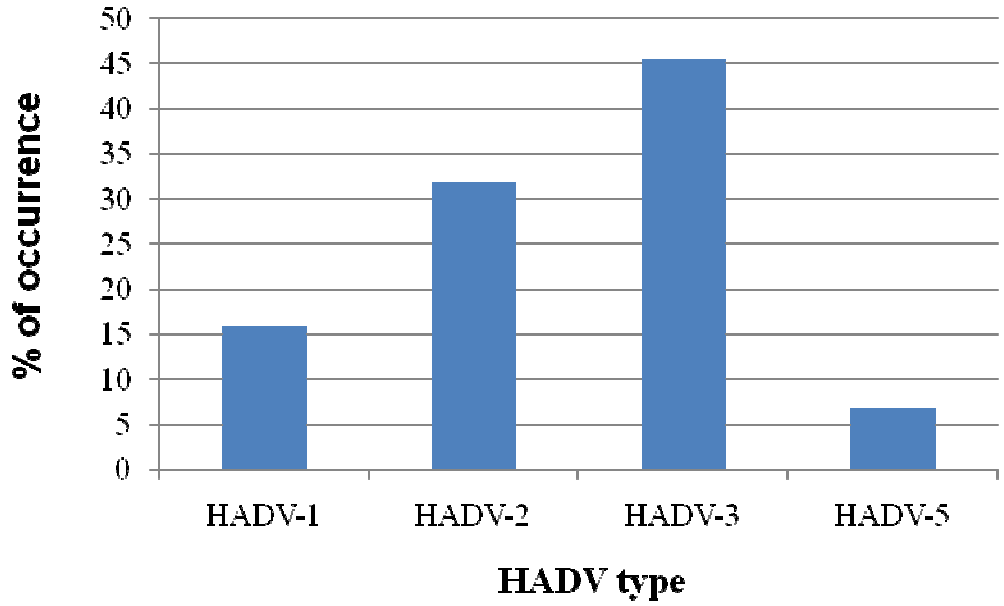

Figure 3.9: HADV serotypes present in southern Palestine. The percentage of each serotype was calculated relatively to the number of total sequenced samples (44 samples).

3.2.3.2 DNA based phylogenetic analysis of southern Palestinian HADV isolates

The Hexon gene sequences of the different Palestinian HADV isolates were subjected to genetic alignment using the MegAlign program (DNASTAR Inc., Madison, WI, USA). In this case, all sequenced Palestinian samples were first subjected to alignment with reference hexon genes of serotypes 1, 2, 3, 5 (figure 3.10). These reference hexon genes were specifically chosen based on the initial alignment results using the blast machinery of the NCBI (see 3.12.3.1). The alignment was performed using the Clustal W method and the alignment parameters mentioned in 2.12.3.1. The result shown in figure 3.10 is a general alignment showing the rough homology of the Palestinian samples' sequences to HADV-1, 2, 3, 5 using the comlete DNA sequences of Palestinian samples and reference hexon gene sequences. This knowledge was used to establish the accurate phylogenetic analysis (see below). As indicated in table 3.4 and clearly shown in figure 3.10, samples from all the years showed homology to HADV-2. Homology to HADV-3 in the Palestinian samples was also seen all over the years with exception to 2009. In case of HADV-1, homology was presented all over the years, with exception of the year 2008. On the other hand, only samples from the year 2005 and 2006 showed homology to HADV-5. Due to the high number of samples subjected to this analysis, different colors were used to refer to the different years, in attempt to make viewing this figure (figure 3.10) easier and more accessible.

The rooted phylogenetic tree shows 2 main branches; A and B. The Adenovirus subgenus C is branched together on branch A, while Adenovirus subgenus B is branched on the main branch B and accordingly the Palestinian samples resembling each subgenus. On branch A1, sample 28 (2006) showed the highest homology (99%) to HADV-2, being branched on the same node. Samples 40 and 11 (2008), sample 66 (2007), sample 1 (2010), sample 58 (2009) and 67 (2007) showed 95-97% homology to HADV-2 and therefore were branched next to the HADV-2 node. Samples 7 and 13 from the year 2005, samples 73, 80 and 83 (2009), sample 26 (2006) and sample 51 (2008), all were sub-branched further below with 90-95% homology to HADV-2.

On branch A2, sample 4 (2005) showed the highest homology (99%) to HADV-5, being branched with at the same node. Samples 3 (year 2005) and 24 (2006) showed 97% homology to HADV-5 and were therefore branched next to the HADV-5 node.

On branch A3, sample 60 (2007) showed the highest homology (99%) to HADV-1, while samples 3 (2010) and 75 (2007) were also highly homolog (97%) to HADV-2. Samples 6 (2005), 85 (2009), 71 (2009) and 31 (2006) were only 93-95% homolog to HADV-2 and therefore branched further away from HADV-2 node.

On main branch B, samples 12 (year 2010), 14 (year 2008), 11 (2010), 8 and 8b (2005), 10 (2010), 7 (2010) and 16 (2008) showed the highest homology (97%) to HADV-3. Samples 23 (2005) and 23 (2006), 29 (2006), 12 (2005), 13 (2007), 9 (2010) showed 95% homology to HADV-3. Samples 14 and 15, 9, 5, 24 and 2 (2005) were less homolog (90-94%) to HADV-3 and therefore branched further below on a different node.

8 and 8b (2005) are actually the same sample being subjected to a second sequencing analysis to obtain better sequencing results.

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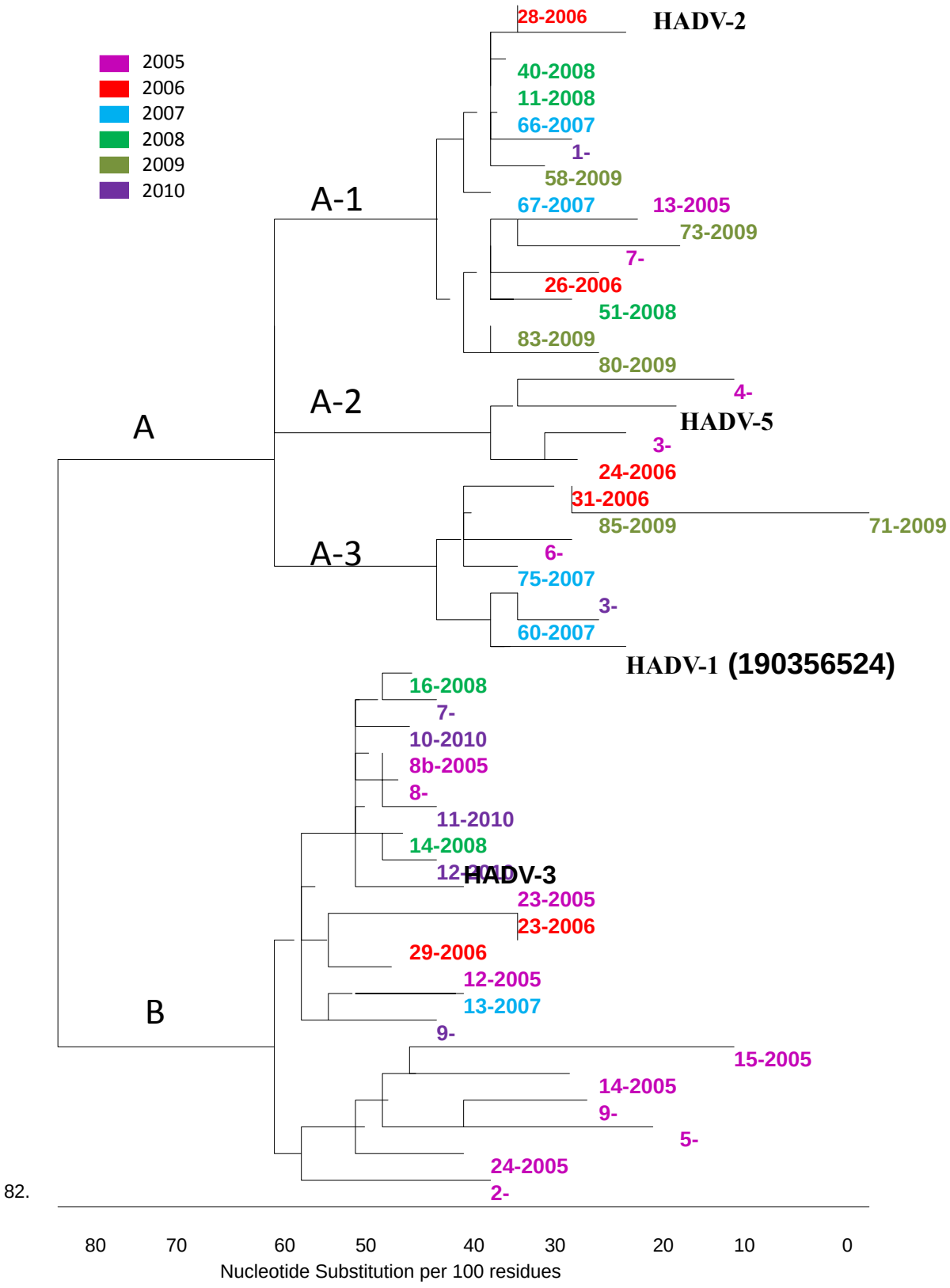
2005
2006
2007
2008
2009
2010
A
A-1
A-2
A-3
B
28-2006
HADV-2
40-2008
11-2008
66-2007
1-
58-2009
67-2007
13-2005
73-2009
7-
26-2006
51-2008
83-2009
80-2009
4-
HADV-5
3-
24-2006
31-2006
85-2009
71-2009
6-
75-2007
3-
60-2007
HADV-1 (190356524)
16-2008
7-
10-2010
8b-2005
8-
11-2010
14-2008
HADV-3
23-2005
23-2006
29-2006
12-2005
13-2007
9-
15-2005
14-2005
9-
5-
24-2005
2-
82.
80
70
60
50
40
30
20
10
0
Nucleotide Substitution per 100 residues

Figure 3.10

Figure 3.10: DNA alignment of the southern Palestinian HADV samples compared with reference serotypes HADV-1, HADV-2, HADV-3 and HADV-5 (ncbi accession numbers in brackets). The phylogenetic tree was generated by using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. Nucleotide substitution per 100 residues reflects one of the bases for the sequence alignment in this figure only.

3.2.3.3 Amino acid molecular typing of Palestinian HADV isolates

As sequences were subjected to nucleotide blast machinery (see above), some sequences showed homology not only to one HADV known serotype, but also to another one or two HADV serotypes. On the other hand, no Palestinian sequence showed a 100% homology to any of the published HADV serotypes. To further investigate this finding, the Palestinian sequences were subjected to amino acid homology analysis as introduced by Crawford-Miksza and Schnurr (Crawford-Miksza and Schnurr, 1996). Hereby, the amino acid sequences of the high variable regions 1-6 (HVR1-6, amino acid position 131 to 331) were subjected to alignment and analysis. Since the genetic differences in the hexon gene are responsible for the different serotypes, the amino acid sequences of the high variable region of the hexon gene reflect the accurate analysis of different human adenovirus genotypes.

The alignment was performed as detailed in 2.12.3.1. Due to the high amount of data in one figure, each year was aligned separately with the reference HADV serotypes. Figure 3.11 shows an example of amino acid alignment for the samples of 2010. As the case for most species, some sequences were highly conserved (in black), other were not conserved (in red). The blue refers to those amino acids, which are indeed different but not on functional basis. All amino acid alignments were performed using the MegAlign DNASTAR program's Clustal W method. Alignment conditions are detailed in 2.12.3.1 and 2.12.3.2.

Figure 3.11: Amino acid alignment of Palestinian HADV samples with reference HADV serotypes using the MegAlign of the DNASTAR program. In black are the absolutely conserved amino acid regions, while red shows the differences and blue the change in amino acid, but not the properties of the amino acid.

The phylogenetic tree was built based on such alignments for each year (see 2.12.3.2). The phylogenetic trees are built based on the sequence distances between the different samples and references calculated by MegAlign. The bootstrapping results are shown directly on each tree branches and nods as calculated by the MegAlign program based on the alignment analysis.

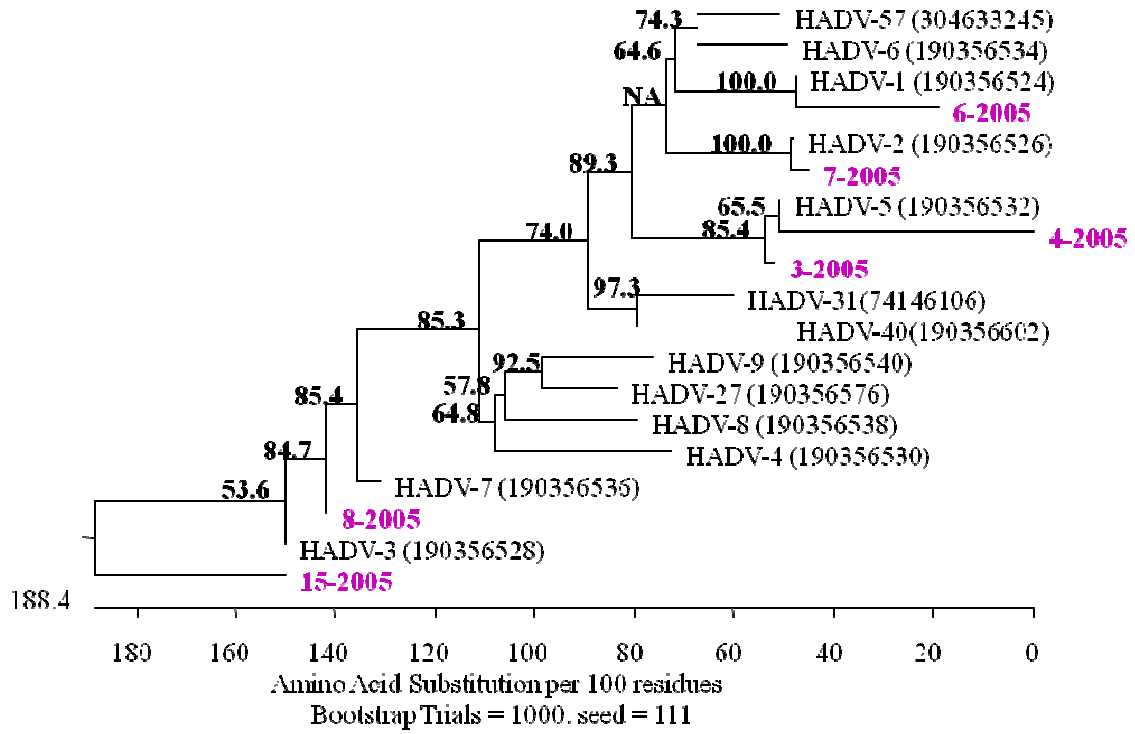

Figure 3.12: Amino acid phylogenetic analysis of the Palestinian HADV samples in the year 2005. The phylogenetic tree was generated by using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. Samples are in light purple, while reference sequences with accession numbers are in black. The bootstrapping numbers are in bold. The nearer the sample is to a reference serotype, the more amino acid homology they have in common.

Year 2005

Phylogenetic analysis of 2005 HADV samples in figure 3.12 showed that they aligned with HADV-1 represented by sample 6-2005, HADV-2 represented by 7-2005, HADV-5 represented by 3-2005 and 4-2005 and HADV-3 represented by 8-2005 and 15-2005. Sample 8-2005 showed high homology to HADV-7, a fact which was not detected using the DNA alignment or the direct blast method. The alignment and so homology between HADV-1 and sample 6-2005, between HADV-2 and 7-2005 was very high strengthened by a perfect bootstrap value of 100.

6-2005 aligned with a lower bootstrap value with HADV-6 (bootstrap value=64.6) and HADV-57 (bootstrap value =74.3). Although 4-2005 showed higher homology with HADV-5 compared to sample 3-2005, its bootstrap value is 65.6 compared to 85.4 bootstrap values for 3-2005. Both, 8-2005 and 15-2005 aligned with HADV-3. 8-2005 showed also homology to HADV-7.

Year 2006

Phylogenetic analysis of 2006 samples showed that they align with HADV-3 represented by 29-2006, HADV-5 represented by 24-2006, HADV-2 represented by 28-2006 and HADV-1 represented by 31-2006. The high bootstrap values on nodes for these samples' alignment confirm the homology. Interestingly, sample 29-2006 shows a further homology to HADV-7, a fact which was not detectable using the initial blast method used above.

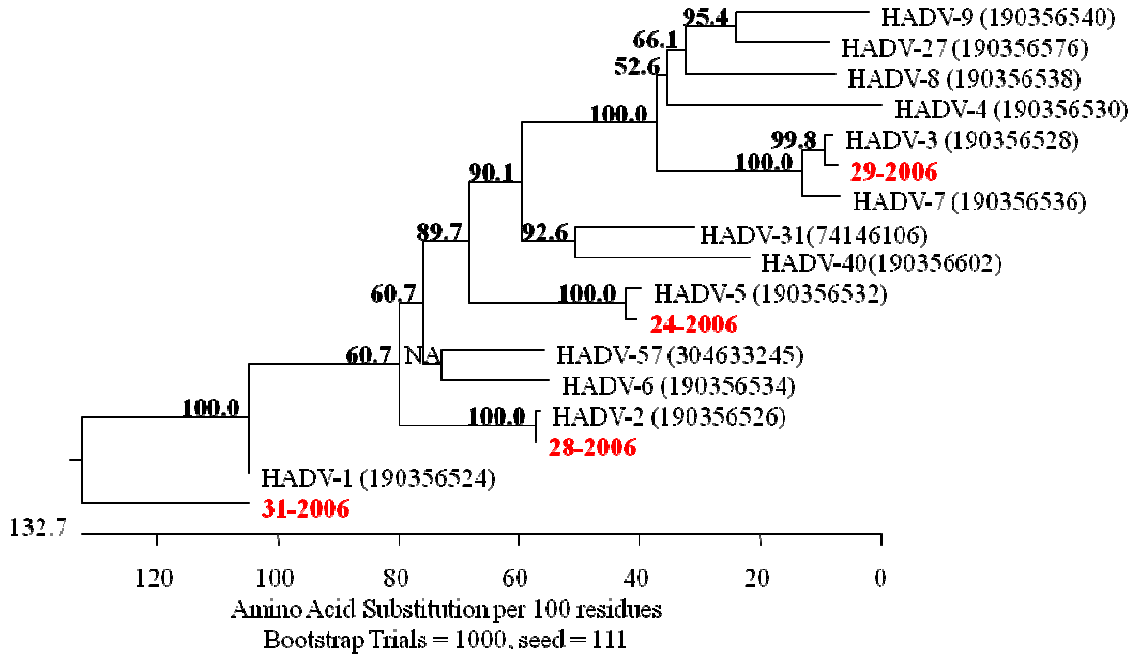

Figure 3.13: Amino acid Phylogenetic analysis of the Palestinian HADV samples in the year 2006. The phylogenetic tree was generated by using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. Samples are in red, while published sequences with accession numbers are in black. The bootstrapping numbers are in bold. The nearer the sample is to a reference serotype, the more amino acid homology they have in common.

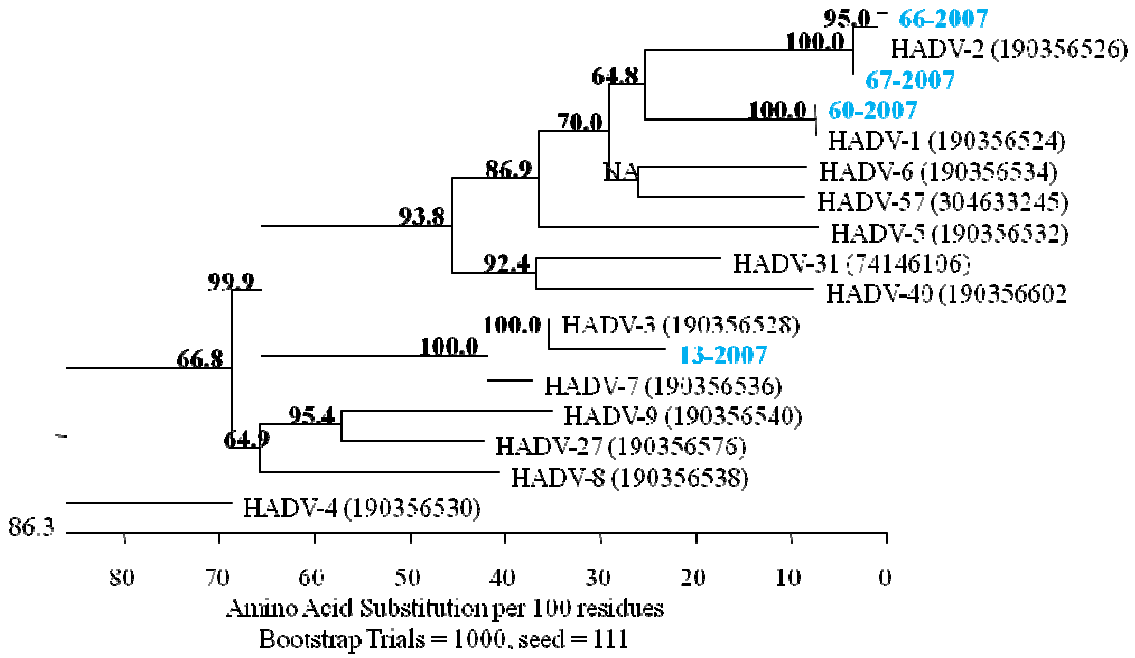

Figure 3.14: Amino acid Phylogenetic analysis of the Palestinian HADV samples in the year 2007. The phylogenetic tree was generated by using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. Samples are in blue, while published sequences with accession numbers are in black. The bootstrapping numbers are in bold. The bootstrapping numbers are in bold. The nearer the sample is to a reference serotype, the more amino acid homology they have in common.

Year 2007

The phylogenetic analysis of HADV 2007 samples shows that they aligned with HADV-2 represented by samples 66-2007 and 67-2007, HADV-1 represented by 60-2007 and HADV-3 represented by 13-2007. The bootstrap values for these alignments on nodes confirm the high homology between the samples and the reference serotypes. Sample 60-2007 shows further homology with serotypes HADV-6 and HADV-57.

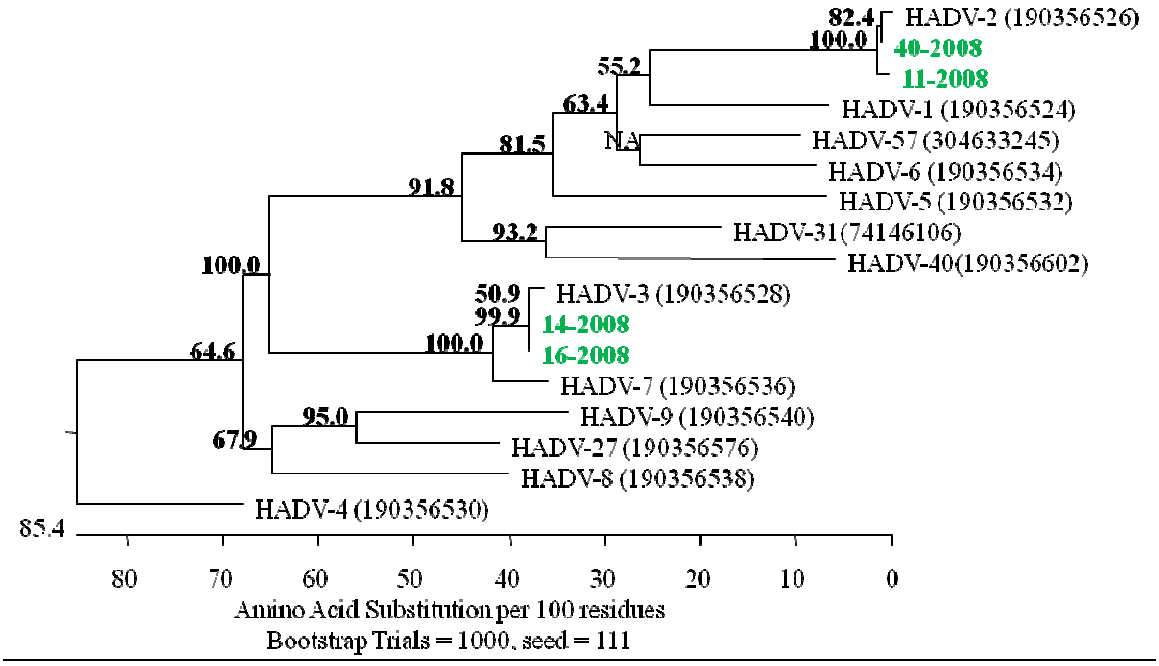

Figure 3.15: Amino acid Phylogenetic analysis of the Palestinian HADV samples in the year 2008. The phylogenetic tree was generated by using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. Samples are in green, while published sequences with accession numbers are in black. The bootstrapping numbers are in bold. The bootstrapping numbers are in bold. The nearer the sample is to a reference serotype, the more amino acid homology they have in common.

Year 2008

The phylogenetic analysis of HADV 2008 samples showed homology with HADV-2 represented by 40-2007 and 11-2007 and with HADV-3 represented by 14-2008 and 16-2008. In both cases though, the homology between the samples was higher than that with the HADV reference serotype as indicated by the bootstrap values. Interestingly, also in this year, the samples, which aligned with HADV-3, show further homology to HADV-7.

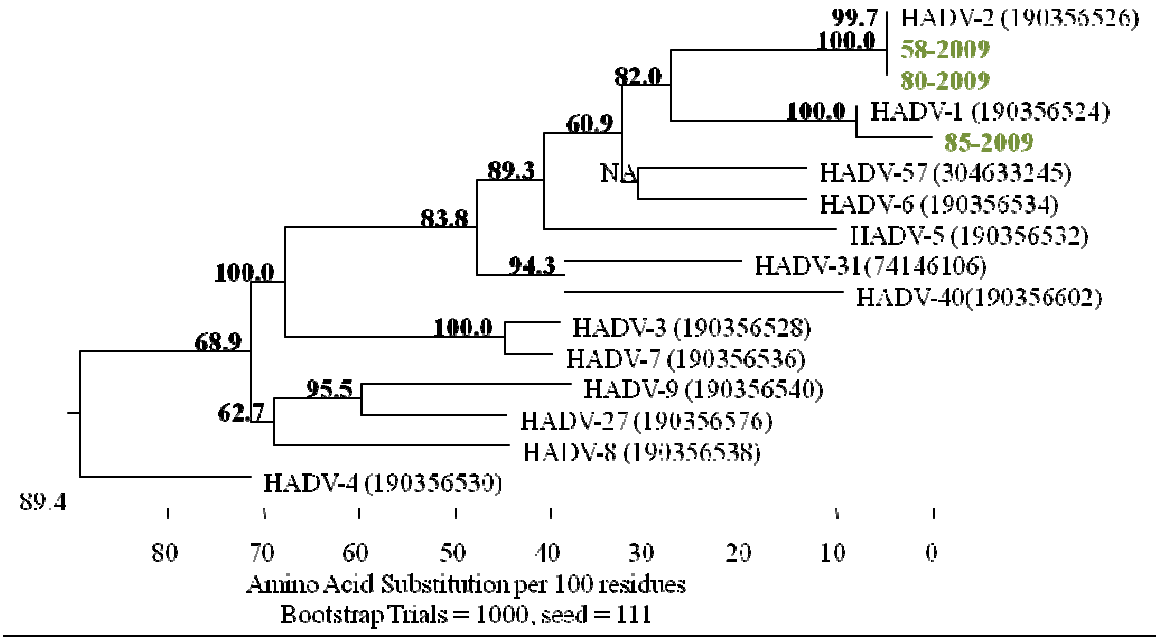

Figure 3.16: Amino acid Phylogenetic analysis of the Palestinian HADV samples in the year 2009. The phylogenetic tree was generated by using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. Samples are in olive, while published sequences with accession numbers are in black. The bootstrapping numbers are in bold.

Year 2009

Phylogenetic analysis of the Palestinian HADV samples in the year 2009 showed that they aligned only with serotypes HADV-2 represented by 58-2009 and 80-2009 and HADV-3 represented by 85-2009. In both cases, the bootstrap values were perfect confirming the homology.

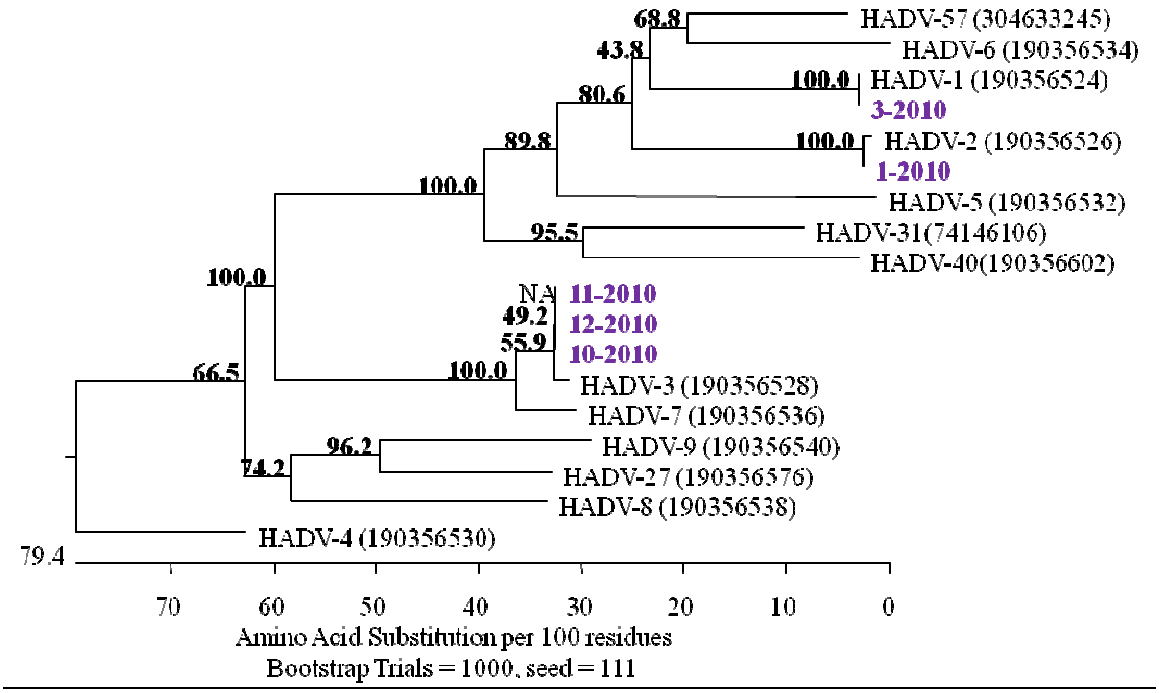

Figure 3.17: Amino acid Phylogenetic analysis of the Palestinian HADV samples in the year 2010. The phylogenetic tree was generated by using the neighbor-joining method (MegAlign-DNASTAR program). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. Samples are in purple, while published sequences with accession numbers are in black. The bootstrapping numbers are in bold. The bootstrapping numbers are in bold. The nearer the sample is to a reference serotype, the more amino acid homology they have in common.

Year 2010

Phylogenetic analysis of the Palestinian HADV samples in the year 2010 showed that they aligned with HADV-1 represented by 3-2010, HADV-2 represented by 1-2010, HADV-2 represented by 1-2010 and HADV-3 represented by 10-2010, 11-2010 and 12-2010. The perfect bootstrap values reflect the confirmation of these homologies. Interestingly, also here, all samples aligned to HADV-3 also aliened with HADV-7.

3.3 Annual Distribution of human adenovirus serotypes

The different serotypes of HADV were not present all over the years equally. While HADV-2 serotype was present all over the years 2005-2010, HADV-1 was present in all years except 2008, HADV-3 was present in all years except 2009 and HADV-5 was present only in 2005 and 2006 (table 3.4).

SEROTYPES	2005	2006	2007	2008	2009	2010
HADV1	+	+	+	_	+	+
HADV2	+	+	+	+	+	+
HADV3	+	+	+	+	_	+
HADV5	+	+	_	_	_	_

Table 3.4: The Adenovirus serotypes circulating in southern Palestine. Sequences resulted from each year's samples were subjected to sequence blast analyses using the NCBI machinery (see above). The different serotypes in each year were documented.

3.4 Seasonal variation of Adenovirus serotypes

In the years 2007 and 2010, HADV-1, HADV-2 and HADV-3 serotypes were present, while only HADV2 and HADV-3, HADV-1 and HADV-2 were present in the years 2008 and 2009 respectively. Generally, all serotypes circulated all over the months each year with higher tendency in winter and spring seasons generally. The seasonal distribution varied between the different serotypes. While HADV-1 circulated mainly in the winter months December and January, HADV-2 circulated mainly in spring month March, followed by autumn months October and November. HADV-3 circulated mainly in spring too, but in the months March and May, followed by the winter months December and January.

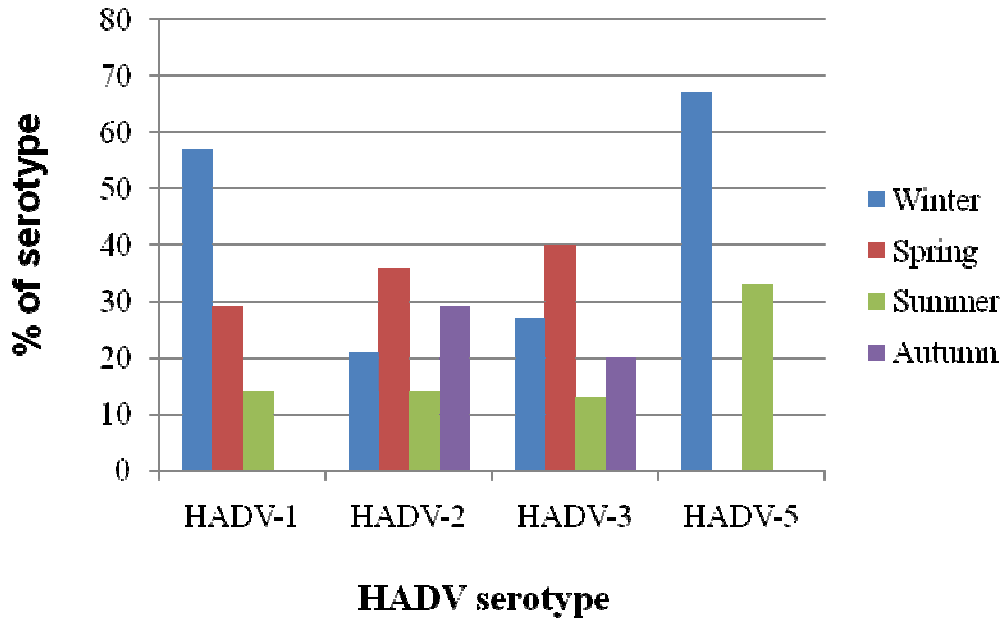

Figure 3.18: Seasonal distribution of Adenovirus serotypes. The total number of the same serotype in each season was calculated in relation to the total number of the same serotype.

3.5 Residency, age and sex distribution of Adenovirus serotypes

All serotypes were found all over southern Palestine, without specific distribution to city, village or refugee camp. In both cities; Bethlehem and Hebron as well as in refugee camps Aida and

Dheisha, serotypes HADV-1, HADV-2 and HADV-3 were present. HADV-1, HADV-2, HADV-3 and HADV-5 were found all over the villages in Bethlehem and Hebron districts.

Also in regards to age group and sex of the children, no further specific distribution of serotypes was found.

4. Discussion

Adenovirus is a significant causative agent of respiratory tract disease in pediatric and adult patients. Numerous outbreaks of acute respiratory infections caused by HADV have been reported during the last decade in many countries (Harley et al., 2001; Ryan et al., 2002; Palomino et al., 2004; Chmielewicz et al., 2005a; Zhang et al., 2006; Mizuta et al., 2006; Chang et al., 2008; Zhu et al., 2009; Esposito et al., 2010; Kandel et al., 2010; lee et al., 2010; Rebelo-de-Andrade et al., 2010). Some outbreaks resulted in high morbidity and fatal death in neonatal and pediatric units, in children admitted to hospitals (Wu et al., 1990; Pinto et al., 1992; Mitchell et al., 2000; Carballal et al., 2002; Louie et al., 2008; Rebelo-de-Andrade et al., 2010), in schools (Zhu et al., 2009) and most strikingly in military camps (van der Veen et al., 1969, Mantyjarvi, 1966; Chmielewicz et al., 2005a) and military hospitals (Lessa et al., 2007).

Presented here are genetic epidemiological data analysis of 338 records of Adenoviruses isolated from childrenresiding southern Palestine between 2005 and 2010. First, epidemiological analysis of Adenovirus was compared with respiratory tract infections Parainfluenza and Influenza A residing the same geographical area. Second, the genetic analysis results of the variable Palestinian Adenovirus serotypes were analyzed accordingly with the epidemiological factors and patients records.

4.1 Residency of infected children

The highest percentage of infections among children in our sample population with Adenovirus, Parainfluenza and Influenza was located in villages with 68%, 72% and 75% respectively. This can be due to the high possibility of contact between children in the village community. Generally, high contact between children is granted by the fact that in most villages, only one or two kindergartens and schools are available. On the other hand, most households are crowded, since the families are bigger than in cities. All these factors raise the possibility for high contact between children overall (Munoz et al., 1998, Lessa et al., 2007). On the other hand,

socioeconomic status of these children residing villages play a crucial role in respiratory tract infections. Adults and children of lower socioeconomic status are known to be at higher risk for a wide range of communicable infectious diseases, especially respiratory infections (Cohen, 1999). In the city, the percentage of occurrence was 21% for Adenovirus, 19 % for Parainfluenza and 15 % for influenza respectively. This may be due to the numerous available Kindergartens and schools, plus smaller household family members compared to villages, which are essential factors for decreased respiratory tract infections.

Surprisingly, the number of cases from refugee camps was the lowest in this study, which can only be explained by the fact that camps' residents rather take advantages of clinical services provided by the UN. This means that the total number of children admitted to the CBH from refugee camp may be small, compared to the number referring to the UN health centers.

The SPSS analysis showed that there is no significant association between Adenovirus infection and district residency as (P value > 0.05[95%CI= 1.7113-1.6558]). In regard to Parainfluenza, there was a difference in distribution of infection according to residency (mean difference=1.57103 [95% CI= 1.6005-1.5415]) and similarly for Influenza (mean difference=1.450 [95 %CI= 1.49-1.410]).

4.2 Age distribution of infected children

The most striking finding was the very small numbers of infection cases involving school age children (age 5 years-11 years). This may suggest that they have already been exposed to the common endemic serotypes of Adenovirus early in life and have thereby established a protective immunity (Cooper et al., 2000). Children younger than 2 years were the most susceptible to respiratory tract infections of Adenovirus with 62% for age group 0-1 and 27% for age group 1.1-2 compared with 11% only in school age children respectively. Adenovirus outbreaks were more likely to occur in young children and infants (Wu et al., 1990; Pinto et al., 1992; Mitchell et al., 2000; Carballal et al., 2002; Louie et al., 2008; Rebelo-de-Andrade et al., 2010).

Parainfluenza infections are most severe in infants and become less severe with age. By school age, most children have been exposed to Parainfluenza virus so most adults have antibodies against Parainfluenza (Karron and Collins, 2007). Similarly, age group 0-1 showed the highest percentage of Parainfluenza infection cases (73.7%). The same was noticed in influenza A infection, the risk of complications is higher in young infants than it is for any other age group (Brankston et al., 2007). In this study, Influenza infection cases among infants were (66%). These findings were confirmed statistically, as the correlation between age and the severity of Adenovirus infection was clearly present (r=0.110), but even more significant for Parainfluenza infection (r=0.127). The lowest significant correlation was in case of Influenza infection showing a low value of Pearson correlation (r=0.018).

4.3 Sex of infected children

This study showed clearly that in our sample males were more likely to be infected with respiratory tract infections Adenovirus, Parainfluenza and Influenza. Male and female ratio was calculated according to the frequency of either case all over the six years period (2005-2010) relatively to the total number of cases. The percentage for the three types of infections were very similar; 62% males in case of Adenovirus infection, 64% in case of Parainfluenza and 64% in case of Influenza respectively. This sex based infection incidences were statistically significant for Adenovirus (mean difference=1.683[95% CI= 1.6005-1.5415]), for Parainfluenza (mean difference=1.57103 [95%CI =1.6005-1.5415]) as well as for Influenza (mean difference= 1.33012 [95%CI= 1.3959-1.3384]) respectively. This finding was previously confirmed for Adenovirus respiratory tract infections (Cooper et al., 2000; Schmitz et al., 1983), however, this phenomenon may vary for specific Adenovirus serotypes (Cooper et al., 2000; see also 4.7)

4.4 Seasonal occurrence of infections

Results for seasonal occurrence of Adenovirus demonstrated that the infection occurred throughout the whole year, but generally it was more significant in the months March to May. The SPSS analysis showed an agreement with these results, as there was a correlation between Adenovirus infections and season of occurrence (P value<0.001). Although there was no clear evidence for seasonal correlation of Adenovirus infection generally, some Adenovirus serotypes correlated specifically to one or two months throughout the year and were also found in some years, but not in others (Cooper et al., 2000; see also section 4.7)

The case was generally different for Influenza A and Parainfluenza, since both occurred seasonally rather than uniformly throughout the year especially for Influenza A (3.5 C and D). These results are consistent with previous studies demonstrated by Chew et al. (Chew et al., 1998). One possible explanation for this is that people are indoors more often during the winter, they are in close contact more often, and this promotes transmission from person to person (Karron and Collins, 2007). Therefore, the SPSS analysis showed a clear mean difference of occurrence between Parainfluenza and season (95%CI=1.5415-1.6005), as well as Influenza A and season (95%CI =1.7156-1.7678).

4.5 Severity of infection, Fever and cough in Adenovirus infected children

The major Adenovirus associated diseases as indicated in medical charts were bronchiolitis and bronchopneumonia (33.3%), upper respiratory tract infection (24.7%) and sepsis (5.3%). Indeed, the most common recognizable clinical presentations of Adenovirus infection are pneumonia, bronchitis/bronchiolitis, conjunctivitis, pharyngoconjunctival fever, and upper respiratory tract infections or common cold symptoms (Wu et al., 1990; Ruuskanen et al., 1992; Singh-Naz et al., 1993; Hemming, 1994; Denny, 1995; Munoz et al., 1998; Palomino et al., 2000; Palomino et al., 2004; Chmielewicz et al., 2005a; Yeung et al., 2009). Interestingly, these symptoms remain similar in different age groups (Cooper et al., 2000).

According to the medical charts, 80% of children admitted to the CBH with Adenovirus infection suffered from fever as a major symptom of lower respiratory disease whereas, 50% only suffered from cough. There was a significant association between fever and Adenovirus infection (P value<0.001 [95%CI= 1.7113-1.6558]) as well as between cough and Adenovirus infection (P value <0.001 [95%CI 1.7113-1.6558]). Cough and fever are common symptoms related to different respiratory tract infections (Chmielewicz et al., 2005a); nevertheless they belong to known clinical features in Adenoviral respiratory tract infections with (Munoz et al., 1998; cooper et al., 2000; Hsieh et al., 2009).

4.6 Medication and treatment of Adenovirus infected children

Viral infections cannot be treated with antibiotics and so Adenovirus infections, however, alternative antibiotic like Clindamiycin and Tetracyclines are sometimes prescribed along with ibuprofen by physicians in extreme cases to help with fever, pain and inflammation. There are very few options of antiviral medications for use in case of Adenovirus infections. Though, certain serotypes of Adenovirus may respond to Ribavirin antiviral therapy in vitro, but most serotypes respond to Cidovir (Morfin et al., 2005; 2009).

In this study, antiviral therapy was not administered in any of the children with adenoviral respiratory tract infection; however, most of these children (70%) have taken Antibiotics as clearly stated in the medical files. In most cases, antibiotics were administered automatically, before the CBH laboratory diagnostic result was reported or even before the children were admitted to the CBH for health care. Antibiotics may be useful in some cases to prevent further complications caused by bacterial infections due to weak immunity during viral infections or in case of viral co-infection with bacterial agent (Franz et al., 2010; Shiley et al., 2010). On the other hand, general prescription of antibiotics before diagnosis or because exact diagnosis is not possible is a common mistake made by most physicians which have led worldwide to more and more resistant bacterial strains and unexpected complications in patients (Rocholl et al., 2004; Lautenbach et al., 2010; Shiley et al., 2010; Pavia, 2011).

4.7 Adenovirus serotypes in Palestine

This study demonstrated that HADV-1, HADV-2, HADV-3 and HADV-5 were the most common serotypes circulating in southern Palestine. The different serotypes were not equally distributed all over the years or all over the months.

Similar genetic epidemiology studies had shown that Adenovirus serotypes HADV-1, 2, 3, 5, 6, and 7 were mainly involved in respiratory tract infections in children in Argentina, Iceland, Chile, Taiwan, Canada, China, Malaysia, Korea, Israel and the USA (Kajon and Wadell, 1992; Johansson et al., 1993; Palomino et al., 2004; Hsieh et al., 2009; Yeung et al., 2009; Zhu et al., 2009; Abd-Jamil et al., 2010; Lee et al., 2010; Mandelboim et al., 2011; Selvaraju et al., 2011). Adenovirus serotypes 1, 2 and 3 were detected in most of these studies, serotype 5, however was detected in Iceland and Taiwan (Johansson et al., 1993; Hsieh et al., 2009). Adenovirus serotypes; HADV-1, 2, 3, 5, 6 and 7 were associated with respiratory disease ($p<0.001$), most consistently with mild upper respiratory tract disease (Louie et al., 2008). These same serotypes are known to be associated with almost 85% of the HADV infections in infants and children (PA, P, 1962).

Although most studies on Adenoviral infections were unable to show a clear evidence for seasonal correlation, specific serotypes correlated to specific seasons (Chew et al., 1998; Cooper et al., 2000; see also 3.3 and 3.4). Also here, HADV-2 and HADV-3 seems to be more frequent in spring and winter seasons.

Of all Adenovirus serotypes detected in southern Palestine, only HADV-3 (subgenus B) was shown in numerous studies to be responsible for outbreaks; in the USA, Taiwan, Korea and Portugal (Ryan et al., 2002; Chang et al., 2008; Lee et al., 2010; Rebelo-de-Andrade, 2010). Fatal Adenovirus serotype 14 (Louie et al., 2008; Lessa et al., 2007) was not detected in any of the Palestinian samples. Although there was no record of possible outbreak in southern Palestine, the serotyping results show clearly that HADV-3 was the most common serotype in the year 2005, since it was detected in 9 samples out of 14. In the year 2010, HADV-3 was detected in 5 samples out of 7. These data may provide evidence for a possible unknown outbreak, since HADV-3 was also reported to be detected in both, outbreaks and sporadic cases (Lee et al., 2010). Some Palestinian samples, which showed high homology to HADV-3, were

also homolog to HADV-7 (see 3.2.3.4). HADV-7 belongs also to subgenus B and was also shown in numerous publications to be responsible for several outbreaks (Kajon et al., 1990; Larranaga et al., 2000; Mitchell et al., 2000; Carballal et al., 2002; Erdman et al., 2002; Ryan et al., 2002; Gray et al., 2005; Rebelo-de-Andrade et al., 2010).

4.8 Phylogenetic tree analysis of Palestinian Adenoviruses

The reference sequences used for phylogenetic analysis represent all known Adenovirus subgenus (A-F). HADV 31 represents subgenus A, HADV-3 and HADV-7 represent subgenus B, HADV-1, 2, 5, 6 and 57 represent subgenus B, HADV-8, 9 and 27 represent subgenus D, HADV-4 represents subgenus E and finally HADV-40 represents subgenus F.

Initially the phylogenetic analysis was performed using the DNA sequences of all samples. This was a general overview of the Palestinian samples, showing their general relevance to reference HADV serotypes. Some previous studies on Adenovirus based the serotyping on Restriction enzyme analysis (Adrian et al., 1986; Niel et al., 1991; Kajon et al., 1996), however more recent studies made use of DNA sequences by simplex or multiplex PCR assays for specific serotypes (Lu and Erdman, 2006; Adhikary et al., 2004; Lee et al., 2010). The second way of genotyping was based on using primers which specifically amplify one or few serotypes and confirmed per sequencing analysis.

However, precise analysis of the Adenovirus genotypes requires the involvement of amino acid sequences of the hexon gene variable region (HVR1-6), better HVR1-6 and HVR-7 (Xu and Erdman, 2001, Zhu et al., 2009; Biere and Schweiger, 2010). This is logic and more convenient, since antigenic characteristic of the HVR resembles the baseline of the necessity of serotyping procedure. Any amino acid substitution event will result in a different antigenic characteristic and probably a new serotype, if the amino acid substituted was critical for the antigenic protein conformation. Applying the amino acid alignment on the Palestinian sequences granted a new

insight into these sequences, their relevance to published reference serotypes and the impact of such data on the long term.

The DNA blast and later the DNA sequence alignment and phylogenetic analysis had shown that many of the Palestinian serotypes, which showed homolog to HADV-1, were also homology to HADV-6 and HADV-57 (all subgenus C). On the other hand, the Amino acid sequence alignment and phylogenetic analysis revealed that most of the Palestinian samples related to HADV-3, were also related to HADV-7. One possible explanation will be that such samples may be a mix of the 3 viruses (HADV-1, HADV-6 and HADV-57) represented mainly by 4 samples (6-2005, 60-2007, 85-2009 and 3-2010) or of the two viruses (HADV-3 ad HADV-7) represented mainly by 8 samples (8-2005, 29-2006, 13-2007, 14-2008, 16-2008, 10-2010, 11-2010 and 12-2010). Indeed, there are many evidences of frequent recombination between Adenoviruses resulting in new serotypes (Lukashev et al., 2008; Seto et al., 2010; Biere and Schweiger, 2010). This fact explains also why so many new serotypes are joining the long list of Adenovirus known serotypes every year. Within a year and a half, needed to accomplish this work, the number of Adenovirus serotypes raised from 51 up to 58 (Lu and Erdman, 2006; Jones et al., 2007; Walsh et al., 2009; Seto et al., 2010; Ishiko and Aoki, 2009; Walsh et al., 2010; Robinson et al., 2011; Liu et al., 2011). It is also noteworthy to mention that many Palestinian serotypes showing high homology to HADV-2, also showed homology to HADV-1, HADV-6 and HADV-57, all subgenus C represented mainly by 9 samples (7-2005, 28-2006, 66-2007, 67-2007, 11-2008, 40-2008, 58-2009, 80-2009 and 1-2010).

Palestinian sequences, which were not distributed on the phylogenetic tree directly at the same final node, where either of the reference published serotypes is distributed, provide evidence for a new recombination of a serotype. This was the case for at least 6 samples; 3-2005, 8-2005, 15-2005, 31-2006, 67-2007 and 11-2008. This assumption will be subjected to further computational and genetic analysis, but is strongly supported by the bootstrapping values calculated by the MegAlign program.

Bootstrap values above 95 at the final node, where the reference serotype and Palestinian serotype is distributed (i.e. samples 6-2005 and 7-2007) refer a very high homology and therefore for the exact concurrent serotype. This means that 6-2005 is 100% HADV-1, while 7-2005 is 100% HADV-2. All cases, where bootstrap values were below 90 refer to high

heterology in the DNA or the amino acid sequencing and so for a yet unknown serotype, which could not be tightly correlated to one of the existing reference serotypes, which represent all the Adenovirus subgenus.

4.9 Recommendations

Rapid diagnostic approaches of respiratory tract infections can be very critical in patient's life especially in outbreaks occasions. Zhu et al (2009) had reported the identification of HADV-11, which was never been correlated earlier with outbreaks or fatal infections, to be responsible for an outbreak in china, which led to death in a school outbreak. This study and discussions of scientific reports presented here emphasize the necessity of developing rapid and accurate diagnostic methods, which enable the identification of the specific bacterial and viral agents causing respiratory tract infections within one day or less. A precise diagnostic should play a major role whether to consider administration of antibiotics, anti viral or no drugs at all.

Generally, most physicians and pediatricians in Palestine hurry up in conclusions and prescribe basically antibiotics for all symptoms correlating to a possible respiratory tract infection. Studies presented here demonstrate that prescription of antibiotics without having the precise diagnosis of an infection may turn out to be negative, rather than a positive effect on a patient. Treatment with antibiotics must be subjected to more tight rules. On the other hand, the economical impact of such unnecessary costs can be prevented and rather invested somewhere else in the health section.

Study data presented here show that antibiotics problem do not seem to be a Palestinian problem only, not even a third world problem. Most industrial countries suffer from over usage of antibiotics, but compared to Palestine and most countries in the third world, rules do not make access to antibiotics from each pharmacy around the corner possible. On the other hand, research and studies in this area had been flourishing in the industrial world, spreading an enormous awareness in those societies among even simple people. All these actions are urgently

needed in Palestine, taking into consideration the extremely cheap prices for antibiotics produced by local companies.

Altogether, we strongly recommend to control access to antibiotics and to receive high priorities from responsible authorities to support molecular epidemiology studies to be used on daily basis and not for research only. Molecular epidemiology data are only then useful, when they can be applied in daily life for the benefit of human being.

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Appendix 1

Children's Relief **Bethlehem**

Caritas Baby Hospital
P.O.Box 84, Bethlehem
Tel. +972 2 275 85 00, Fax +972 2 275 85 01
info@crb-mail.org, www.childrens-relief-bethlehem.org
Arab Bank, Bethlehem, Acct. 702200

19-03-2010

Al-Quds University
Department of Public Health
Abu-Dies
Jerusalem

Dear Sir/Madam,

This letter is written on behalf of Mrs. Lina Qurei, whom is currently doing her Master's thesis on the "Molecular epidemiology of Adenovirus circulating in southern Palestine". Lina submitted a request to Caritas Baby Hospital Medical Research Committee (MRC) to get permission to do the following:

1. Collect statistical data on the epidemiology of respiratory viral infections seen at Caritas Baby Hospital since 2005.
2. Receive archive nasopharyngeal samples in order to do molecular genotyping of adenovirus.
3. Review the charts of patients infected with adenovirus in order to collect clinical information about patient's symptoms and demographics.

After the Caritas MRC reviewed and approved Lina's thesis proposal and after receiving the signed patient confidentially letter, the MRC granted Lina permission to receive the above mentioned items.

Please do not hesitate to contact us for further information.

CARITAS BABY HOSPITAL BETHLEHEM

Musa Hindiyeh, PhD, D (ABMM); MT (ASCP)
Director: Clinical Laboratory and Infection Control
Head: Caritas Baby Hospital Medical Research Committee
Caritas baby Hospital
Bethlehem
Palestine

Appendix 2

Appendix

Southern palestine regions which were included in this study:

A` roub c

A` sakreh

A`yda camp

Abu Njeim

Abu njem

Abu Suhweile

AdhDhahiriye

Aida c

Al Masara

Al Askar

Al Doha

Al Miniya

Al Rashaideh

Al Shawawre

Al Walajeh

Alkhader

AnNajade

ArRihiye

Arab arrashayde

Ash shuyukh

Azzeh c

BaniNa`im

Battir

Behtlehem

BeitFajjar

Beit Anan

BeitAwwa

Beitkahil

BeitUla

BeitUmmar

Bietsahour

BietTa`mirash

Deheisheh c

DeirSamet

Doura

E`beidiyeh

El Doha

Fawwar c

Halhoul

Harmalah

Hebron

Husan

Idna

Jaba`

Joretelsham

Kharas

KhashemAl Karem

Kisan

MarahIma`ll

MarahRabal

Nahhalin

Nouba

Ort Alshama`

Rakhma

Sa`ir

Samou

Samu

Sheokh

Sourif

Ta`amreh

Taffoh

Taffuh

Tarqumya

Thahriyeh

Tqou

Umm lasfa

Umm Salamona

Urtas

Urtas

Wad El Nees

Wad Foukin

Wad Rahhal

Yatta

Za`tara