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Epidemic Kerato-Conjunctivitis; New Era of Required Clinical Practices due to the Emergence of Novel Recombinant Types in *Human mastadenovirus D*

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Abstract

Adenoviral epidemic keratoconjunctivitis (EKC) is mainly caused by specific types in *Human mastadenovirus B*, *H. mastadenovirus D* and *H. mastadenovirus E*. However, the most genetically diverse types and more severe EKC cases are caused by types 8, 37 and 64 (formerly known as 19a) in *H. mastadenovirus D* (HAdVD). The recent characterization of novel types in this species has highlighted the recombinant nature of adenovirus as an evolutionary strategy. Among the more than 30 novel HAdV types with recombinant origins, types 53, 54 and 56, are associated with EKC and have become novel sources of yearly infections in Japan. In many cases, these novel types have been misidentified because of the similarity to the recombinant parents, despite their novel conditions different to the preexisting types associated with EKC. Due to the structural and infectious properties of adenovirus, in Japan nosocomial infections are a frequent source of health risks and economical losses. Currently, only symptomatic treatment is available for adenoviral infections; therefore, proper and effective identification of adenoviral EKC is the only available measure to contain the infection and prevent infectious outbreaks. The understanding of the origins and characteristics of the novel adenoviral types is required for the timely identification and treatment of the ocular infections by these novel adenoviral types.

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Clinical Symptoms and Managements of Adenoviral Epidemic Keratoconjunctivitis

Human mastadenovirus (HAdV) strains are the source of multiple infections in human populations worldwide including ocular infections with a broad range of severity. Epidemic keratoconjunctivitis (EKC) is a major cause of ocular morbidity in developed and developing countries [1]. Adenoviral conjunctivitis infections are caused mainly by HAdV-3 (in HAdV-B), -4 (in HAdV-E), -8, -19a (renamed 64, see below), -37, -53, -54, and -56 (all in HAdV-D) [2-6]. The first isolation of an adenovirus in an EKC case was performed by Jawetz (UCSF) from a patient in the US who was returning from Asia in 1954 [7]. For over half a century, the adenoviral serotypes recognized to cause frequent and contagious cases of EKC were 8, 19a and 37 with well-defined pathologies [8]. The frequency of reported cases produced by the novel adenoviral types 53, 54 and 56 has been steadily increasing after their first report in Japan [9]. In the current study, we review the main characteristics of the novel types characterized as cause of EKC. The EKC are characterized by outbreaks and the development of corneal opacities and hazy vision, which can persist for several months or even years [1,10]. The incubation period varies between 2 and 11 days; patients develop conjunctivitis 7 to 9 days after their exposition to the contagious source. Both eyes are easily infected because of the strong infectivity in adenoviral EKC, but symptoms of the first eye are generally more intense. The common symptoms of the infections include severe hyperemia, diffuse infiltration, papillary hypertrophy, regional lymphadenopathy in most cases, mild swelling of the preauricular nodes, lacrimation, follicular conjunctivitis and formation of multiple subepithelial corneal infiltrates (MSI) and a pseudomembrane [1,8,11]. If the infection reaches the cornea, superficial keratitis, corneal erosion, ulceration occurs and multiple spots of cloudiness under the corneal epithelium can last several years. After analyzing multiple clinical cases, it has been suggested acute bilateral follicular conjunctivitis with intrafamilial infection or

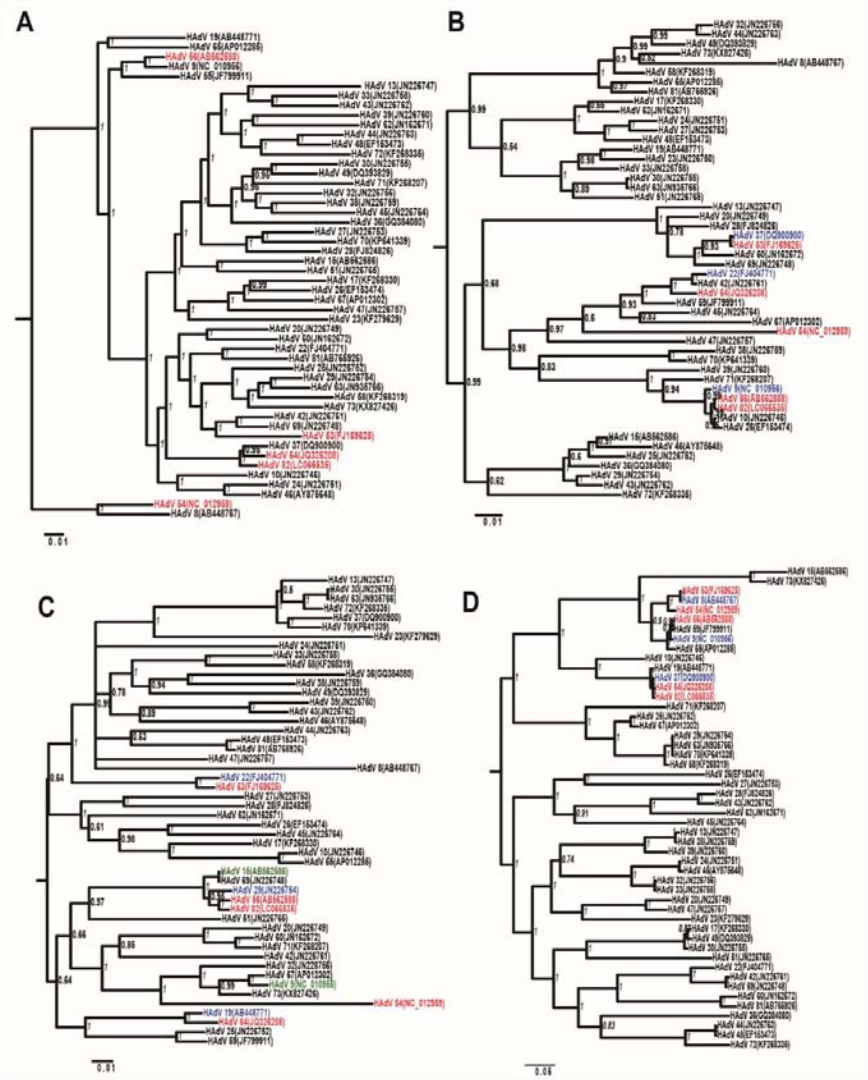

Figure 1: Bayesian inferred phylogenetic trees for *Human mastadenovirus D*. The phylogenetic trees for the types in *H. mastadenovirus D* using the whole genome (A) and encoding regions for penton base (B), hexon (C) and fiber (D), were inferred with Bayesian approaches in MrBayes using a general time-reversible evolutionary model allowing for invariant sites and heterogeneity among sites modeled with a gamma distribution (GTR+G+I). Posterior probability for the branches is shown next to them (i.e. statistical support). Novel types are highlighted in red and types clustering with the novel types are highlighted in blue. In addition, types detected to be related to the novel type are colored in green.

MSI, or both, are strong indicator of adenoviral conjunctivitis in the early stage of infections. Nevertheless, other infectious agents related to conjunctivitis also exhibit similar symptoms and difficult their distinction and subsequent treatment, for example *Chlamydia*, Herpes simplex virus and allergic conjunctivitis [11]. In adenoviral EKC, a small bleeding point occurs in the palpebral conjunctiva, and it can be distinguished from the small bleeding point of the bulbar conjunctiva of acute hemorrhagic conjunctivitis by *Enterovirus* type 70 or coxsackievirus type 24 mutant strains. Pseudomembranous conjunctivitis in neonates and infants is often caused by *Chlamydia*.

In general, there is no effective treatment against adenovirus infection. If inflammation or infiltration to the cornea is detected, it is recommended to use eye drops of anti-inflammatory agents or corticosteroids. Pseudomembranous conjunctivitis is also frequently observed in infants with EKC, which may be possibly because their conjunctiva epithelial cell layer is still in developing process. In EKC cases caused by adenoviral types, pseudomembranous conjunctivitis can be mixed with streptococci infections that lead to corneal perforation; therefore, the application of antibiotics should be supplied carefully. Newborns and infants are in risk of mixed infections with bacteria, therefore, eye drops of antibacterial agents should be applied. It is also highly recommended the disposal of the secretions of patients with ocular infections, proper hand washing and disinfection of patients.

Transmission and Epidemiology

HAdVs are non-enveloped viruses with icosahedral capsids and linear double-stranded DNA genomes spanning between 34 and 36 kbp [12]. Adenoviral structural characteristics allow virions to remain infectious for long periods of time outside hosts and highly resistant to various frequently used disinfectant compounds [13]. These viral properties allow the spread of nosocomial EKC outbreaks by unintended direct contact with contaminated medical equipment. Due to the viral stability outside of infected cells, the virus can survive for extended periods of time in different surfaces [13]. It has been suggested infectious virus can be transmitted by clinicians and nurses by touching towels contaminated with viruses in hospitals, clinics,

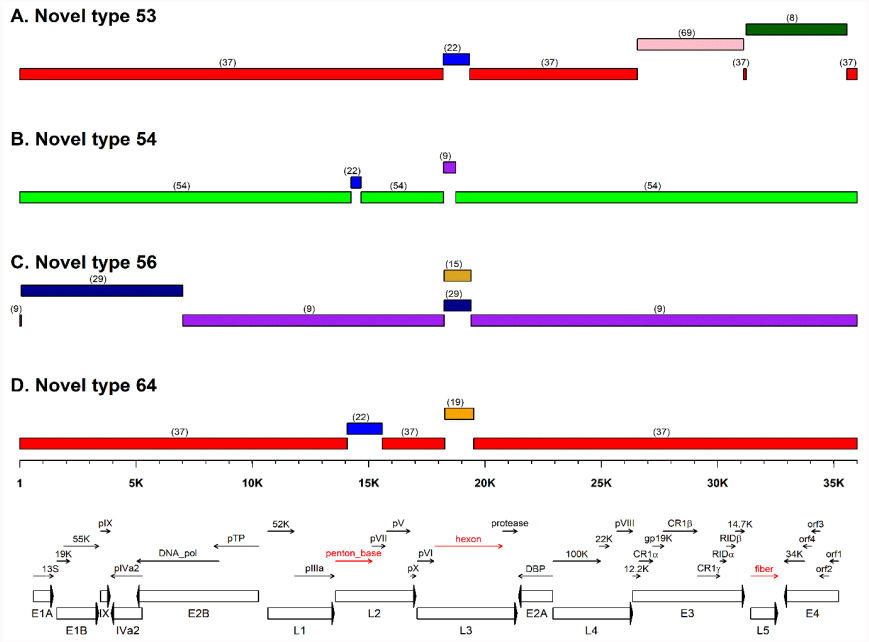

Figure 2: Putative recombinant origin of genomic regions in novel types. Genomic distribution of recombined regions for novel types 53 (A), 54 (B), 56 (C) and 64 (D). The boundaries of the putatively recombined regions were determined by recombination Detection Program (RDP). The putative recombinant parent of the recombined block is shown between parentheses. In the bottom of the diagram, the annotation of the *H. mastadenovirus D* genome is shown as reference. The encoding regions corresponding to Penton Base, Hexon and Fiber (PHF) are highlighted in red. Shown positions are relative to the alignment.

workplaces and homes. As the eyedrop bottles can get contaminated with the virus, it is advised to be careful not to use eye drops of different patients. Also, it is suggested the sterilization with autoclave of contaminated hospital instruments, or disinfection with alcohol, iodine, or other compounds effective against adenovirus. Although there is no difference in frequency by age, it spreads mainly in active adults. In recent serological investigations, the antibody prevalence rate for workers in eye clinics in Japan is less than 30% for type 8, 10% for types 19 and 37 and 5% for the new types. According to the survey of occurrence trend after the Infectious Disease Law enforcement, reports from approximately 600 ophthalmic fixed points nationwide in Japan, 23,000 to 31,000 EKC cases were reported per year [14]. Peaks are seen at the 34th week mainly around August as the summer season ends, but in some areas hospital infections occur from emergency patients even in winter. In addition, adenoviral infections manifest as severe complications in immune-compromised patients such as children, transplant recipients and AIDS patients [15]. Nosocomial infections require the health center to be closed to reduce the risk of EKC or further medical complications, which represents economical losses. The ever-present possibility of adenoviral contamination and opportunistic adenoviral infections creates a constant risk for surgeries. In addition, the possibility of asymptomatic and chronic infections could be the source of new outbreaks.

Emergence of Recombinant Novel Types and Transition to the New Typing Method, and Nomenclature of Adenovirus

For many years the type numbers were assigned based on serological analysis, leading to name them as serotypes from 1 to 51 [16]. However, the new sequencing technologies open the opportunities to better characterize the variants of the existing serotypes and revealed the recombinant properties of adenoviral divergence evolution [17]. Therefore, novel types are denominated also as genotypes due to their characterization based on their genome properties [18]. Almost all novel genotype numbers assigned above 51 were characterized by phylogenomic analysis, with exception of type 54 that was demonstrated to evidence a different serotype to other existing serotypes. Currently, there are above 80 types, including the 51 serotypes, and classified into seven species, HAdV-A to -G, on the basis of nucleotide and deduced amino acid sequences [19]. The *H. mastadenovirus D* is the most diverse species in the genus with above 50 types and many of them evidencing recombinant origins [20]. It is suggested the variation in the antigenic determinants, i.e., penton base, hexon and fiber, created by the recombination events is the source of combinations that escape the acquired immunity in patients. The recombinant origin of the novel types related with EKC is also source of variation on the severity of the cases and the seroprevalence in the population against the recombinant parents and the novel types [20]. The epitope determinants in HAdV are located mostly in the hexon protein, but also with a lower determinant effect in the other major capsid proteins penton base and fiber [21]. Despite the genomic regions encoding these capsid proteins are hotspots of recombination events and source of many novel types, other recombined genomic regions are also source of variation between types. Other important recombination hotspots are located in the E1, E3 and E4 transcriptional regions, which encode proteins in charge of modulating the viral replication process and host immune response [22,23]. These regions have been shown to demonstrate phylogenetic correlation, i.e., coevolution, despite they are target of frequent recombination. The comparison of recombinant types with the putative recombinant parents allows exploring possible genomic characteristics behind the symptoms observed in the different pathologies due to adenoviral infections. Recent genomic studies comparing proteins and sequences of types related to EKC and phylogenetically close-related types unrelated to EKC have pointed to fiber and proteins encoded in E3 as possible culprits of the

severe cases of adenoviral EKC. In the case of the fiber protein, EKC types have fiber knobs with amino acid compositions evidencing unusually high isoelectric point, and enabling ionic interactions with cell-surface sialic acid that works as cellular receptors in ocular cells [24,25]. On the other hand, the E3:CR1β is shown to be exported from the infected cell to attach non-infected leukocyte receptors and disrupt their immune-related function [26].

Detection of Novel Types in Ocular Infections

Viral isolation is performed by culturing on A549 (human alveolar basal epithelial adenocarcinoma cells) the adenovirus from ocular samples such as tears or conjunctival scrapings. The isolated products are used to identify the type by neutralization reaction or partial sequencing of epitope determinants. However, this process requires experienced management and time, because some types can require longer periods to obtain noticeable Cytopathic Effect (CPE) due to the cells used for the culture and the initial viral load in the sample. Due to the long time required for the isolation, identification and type identification the of adenoviral infections, a commercial kit using monoclonal antibody targeting the hexon has been devised to provide a quicker identification of adenoviral infections. However, this kit still evidences limitations related to the number of virions in the sample and the stage of the infection. An alternative diagnostic method is by directly sequencing from the swab samples with nearly universal primers [27-29]. The different discoveries of the novel types have followed a similar pattern. After the identification of an adenoviral agent in an infection, the following typing process of the strain pointed to contradictory results in either serology or partial sequencing of the major epitope determinants. A subsequent phylogenetic and recombination analysis to resolve the identity of the strain sequence proved the recombinant origin of most of them [3-6,17,30]. A program suggested for the recombination analysis is the recombination detection program (RDP) because of the statistical support that it provides for detected recombination signals [31]. The novel types are referred by the distinct combination of types in the penton base, hexon and fiber, referred as PHF (Figure 1). The type on each protein is identified by the closest type clustered in the phylogenetic tree for that protein. The characteristics, geographical location and number of recombination events that gave origin to each novel type are different. Nevertheless, some common characteristics are shared by these novel types. For instance, the origin of these types required events of co-infection between different types, which has been demonstrated for adenoviral respiratory and ocular infections [32]. In addition, the novel types were inferred to have evolved from multiple recombination events [33]; furthermore, some so-called intermediate forms of the recombinant parents have been demonstrated to be source of infections on their own and demonstrate the dynamism of the circulating EKC types.

Novel Adenovirus Type 53 (P37H22F8)

The first formal report of Japanese type 53 was from EKC cases in 2003 isolated in Matsuyama and reported in 2008. The comparison of partial sequences for hexon and fiber suggested a hybrid between types 22, 37 and type 8 (Figure 1). A similar German report involved for the first time type 22 in EKC in some German cases in 2005 [34], and a subsequent phylogenetic analysis arrived to the same conclusion as the Japanese report involving types 22, 37 and 8 in the recombinant origin of the type 53 (Figure 2) [35]. It is noteworthy that type 22 was firstly isolated from an infant with trachoma [36]. On the other hand, a subsequent study fully sequenced and analyzed the genomes of other type 53 and 53-alike strains isolated in 1987, 1989 and 1995. A recombination analysis of these sequences demonstrated that the 53-alike strains were intermediate recombinant steps between type 22/37 and the currently circulating type 53, which fiber encoding region is identical to the one in type 8. In a recent study based on clinical observations, it was reported infections by type 53 are more frequently related to milder infections than other types such as type 8 and 37.

Novel Adenovirus Type 54 (P54H54F8)

The first report of type 54 comes from a nosocomial outbreak at a university hospital in Kobe, Japan on 2000. The isolated strains of this type were non-typeable by existing neutralizing antisera against EKC types; moreover, testing this type with serum raised against other type's demonstrated type 54 is a novel serotype. In contrast, type 53 was a recombinant product that exhibits mixed serological characteristics against types 37, 22 and 8 [5,34]. Following the initial isolation, more cases were found across Japan and have been frequently reported in EKC cases even when the most similar type 8 decreased in report frequency. This type has been limited almost exclusively to Japan, with a reported exception in Greece based on partial sequencing of the hexon encoding region [37]. The sequences from clinical isolates of type 54 form a monophyletic cluster with type 8, which is the most frequently related to severe cases among the EKC-related types. Interestingly, the serological analysis only showed some reaction for antiserum against type 8 and in lower measure with type 9. The genome sequence of both types share > 95% similarity along the genome with the highest divergence found at the genomic regions encoding penton base and hexon, which have been suggested to evidence some form of ancient recombination events (Figure 2). Despite the monophyletic clustering of these types and overall similarity across the genome, it is still unclear the reason behind the lack of reports or possible misclassified EKC cases relatable to this type prior to 2000.

Novel Adenovirus Type 56 (P26H15F9)

Type 56 was independently and simultaneously reported from cases in France and Japan. The cases in France corresponded to a respiratory neonatal fatality with subsequent conjunctivitis in the health care worker who cared for the child on 2009 [30]. On the other hand, the report in Japan corresponded to 11 cases reported across the country in 2008 that evidenced cases of EKC. The recombination analysis of the sequences demonstrated a hybrid origin involving types 26, 15 or 29 and type 9. The serological analysis of this type showed cross reaction with type 9, 15 and 29. A comparison of type 15 and 29 suggests these two serotypes share high similarity on the hexon protein, explaining the hybrid results on the serological analysis. The number of EKC cases due to infections by this type has been increasing recently across Japan. Interestingly, adenoviral ocular infections cases by an intermediate type between type 15 and 9 were reported in Europe in 1968 and in USA during 1970-1980 [38,39], suggesting this type originated long ago and passed undetected or mistyped all these years.

Novel Adenovirus Type 64 (P22H19F37)

The name of type 64 was given to the previously named type 19a to recognize the recombinant origin of this type and its independence from type 19. Type 19 was firstly reported in 1955 from a case of trachoma in Saudi Arabia [40]. On the other hand, a strain with similar characteristics in serum neutralization but a strikingly

distinctive restriction enzyme pattern was isolated 20 years later from EKC cases [41]. Therefore, following the naming convention on that time, the serotype was assigned and a letter representing the distinctive enzyme restriction pattern was assigned to this strain as 19a. However, recombination and phylogenetic analyses on each protein, as suggested above, demonstrated the penton base and hexon encoding regions are recombinant areas with higher similarity to type 22 and 19, respectively, than to type 37, which is the putative origin of the rest of the genome [42].

Other Emergent Ocular-Infectious Agents

The advent of cheaper and more efficient sequence technologies has allowed bettering characterizing strains with inconsistencies on the typing of hexon and fiber, as the major determinants of the type. Recent cases of ocular infections have been characterized as the product of novel recombinant types between already circulating EKC-related types. A recombinant type between types 56 and 37 was isolated in 2011 from EKC cases in Osaka, Japan. Named type 82 with accession number in GenBank as LC066535 and involving a recombination event between types 37 and the novel type 56 and affecting the genomic area encoding penton base and hexon. The nomenclature of PHF is P56H15F37. Careful surveillance is required to distinguish this novel type from type 56 and tracking the pathological differences introduced by the recombination event. On the other hand, another recombinant type from the Japanese region of Kumamoto was recently isolated. This novel strain evidenced a recombination event affecting the genomic region encoding hexon, with the putative minor parent as 64 and the rest of the genome similar to type 53. Interestingly, the phylogenetic analysis strongly suggested the recombination event was between types 53 and 64 rather than between the parents of these already recombinant types (manuscript in preparation). This novel strain is a clear example of the risk to the health of the community introduced by multiple types circulating and the increasing chance of recombination events leading to more contagious or severe recombinant strains.

Final Remarks

The proper identification and surveillance of the novel types will lead to the better understanding of the epidemiological and virologic characteristics behind EKC. Also, better characterization of adenoviral EKC is expected to lead to the development of better prevention and treatment practices. The current nomenclature system for the adenoviral types considers the similarity on the genomic regions encoding the penton base, hexon and fiber (PHF) to distinguish between the different combinations of the types. However, it is important to keep in mind that more than 30 proteins are coded in the rest of the genome and recombination events affect them with different frequency and with different effects on the associated pathologies. Particularly, the E3 transcriptional region has received attention from the research community due to the multiple functions in the encoded proteins as modulators of the host immune reaction.

The consistent conclusion among the reports of these novel types is the recombinant nature of HAdV-D as an evolutionary strategy. The evolution of adenovirus creates diverged types that continue escaping the immune response acquired by the host populations. The proper surveillance and careful monitoring of the differences among the circulating infectious types will lead to the better understanding of the evolutionary forces and host conditions driving the type-divergence and infectious spreading of these infectious agents. Likewise, the proper surveillance and risk-prediction of the adenoviral infections is expected to reduce the negative impact of nosocomial outbreaks.

Finally, the growing variation and differences between all types associated with EKC present research opportunities to effectively establish the genomic variations that determine the severity of the adenoviral infections. The absence of a proper animal model has difficulties the further research of the different stages or the clinical conditions of EKC infections. Therefore, the combinatorial nature of the reported recombinant types and the variation in the severity of symptoms between types create research conditions to better hypothesize and test the regions related with EKC. A better understanding of the cellular pathways targeted by the adenoviral proteins is expected to lead to possible treatments soon.

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