”Pink Eyes”: Confirmation of Pathogens and Investigation and Analysis of Molecular Epidemiology

Tsuey-Li Lin, Chaio-Wei Huang, Chiu-Chu Hsu, Yi-Syue Li, Ying-Chen Lin, Ho-Sheng Wu, Li-Li Lee

Center for Research and Diagnostics, Centers for Disease Control, Department of Health

From Chinese version, pp,254-268

Abstract

An outbreak of “pink eyes” occurred in Taiwan in October 2007, starting in Keelung City and Yunlin County, followed by other areas in Taiwan. A total of 16,047 cases were reported as of October 19, 2007. Ninety-one cases had clinical samples taken. Isolation and identification of pathogens were done by Taiwan CDC. The 91 cases lived in Taipei City, Keelung City, Taipei County, Yunlin County, and Chiayi County. Male to female ratio was 1.1:1. The youngest case was 2-month-old, and the eldest was 86-years-old. Multiple methods were used in the lab for identification and confirmation of the pathogen. Coxsackievirus A24 variant (CA24v) was identified as the pathogen of this “pink eyes” outbreak, which should be called as acute hemorrhagic conjunctivitis (AHC). Molecular epidemiology analyses showed that the genotype of the pathogen was cluster 6 of genotype III, similar to the AHC strain isolated in Singapore in 2005. The strain appeared in Taiwan after 1985 in different years, and this outbreak was the first one in Keelung City after 1994, which also spread toward southern part of the

Received: Nov 29, 2007; Accepted: March 5 2008.

Correspondence author: Tsuey-Li Lin; Address: No.161, Kun-Yang Street, Taipei, Taiwan
E-mail: tllin@cdc.gov.tw

island. The outbreak ended within a month. Health authorities had used multiple prevention strategies, including media advertisement, education in schools, cooperation of medical centers, and efforts of disease prevention personnel. Hand washing, stop sharing towels, decrease exposure in public areas including schools and offices by people having symptoms, and strengthening personal hygiene are effective ways to block or decrease transmission of the pathogen. In addition, the outbreak occurred close to fall, when outbreaks of enteroviruses are not as strong as in summer. From 1986 to 2003, lineages of CA24v had a close relationship to the time of outbreaks. Usually viruses were in the same lineages in two consecutive years. We should strengthen reporting and monitoring of "pink eyes" and its pathogens to prevent another outbreak.

Keywords: pink eyes, coxsacikevirus A24 variant (CA24v), acute hemorrhagic conjunctivitis (AHC), genotype

Introduction

Acute haemorrhagic conjunctivitis (AHC) is a highly contageous eye disease, first reported in 1969 in Ghana. Clinical symptoms include sudden onset pain, swelling, pink eyes, conjunctival hemorrhage, and tearing. One of the eyes may have the symptoms in 6-12 hours, and in 80% of patients, the other eye will have similar symptoms in 24 hours [1]. In addition to adenovirus, two serotypes of enterovirus could produce similar symptoms: enterovirus 70 (EV70) and coxsacikevirus A24 variant (CA24v). CA24v was first isolated in 1970 in an AHC outbreak in Singapore. Fifteen years since then, the transmission was limited to southeastern Asia and India. It spread to Japan, Taiwan, USA, parts of Europe, and countries in South Africa in 1985. Brazil

had outbreaks in 1987, and Puerto Rico, Ghana, and Nigeria had outbreaks in 1988. Korea had outbreaks in 2002, and Tunisia, Guyana, West Indies, and Brazil had outbreaks in 2003. Singapore had outbreaks in 2005 [2-5]. In addition to eye symptoms, the pathogens could also be isolated from healthy individuals, and patients presenting with of the common cold, diarrhea, and acute paralysis [5].

CA24v first appeared in Taiwan in 1985 [6]. It caused AHC in 1985-1986, 1988-1989, 1990-1991, and 1994. In the following years most of the cases were sporadic until the outbreak in 2007 [7].

The 3C gene is used to trace and analyze evolution and spreading of CA24v in molecular epidemiology, and CA24v can be categorized into three genotypes (I, II, and III). Genotype I and II were isolated in Singapore, Hong Kong, and Thailand in 1970. Genotype III was isolated in 1985-2005 in Japan, Taiwan, Ghana, Hong Kong, Thailand, Singapore, and Pakistan. Genotype III can be further separated into six subgenotypes [4, 5, 6, 8, 9].

Material and methods

1. Samples

Samples were gathered from 91 patients who presented with conjunctivitis, secretion and pain in the eyes, and lived in areas of pink eyes outbreaks. Samples were gathered by eye swabs and sent to the CDC for pathogen isolation and identification

2. Virus isolation [10-11]

Preservatives of eye swabs were spun for 15 minutes at 3000 rpm. Supernatants were then inoculated onto RD, HEp－2c, and HeLa cell lines and cultured at 36℃ in 5% CO_2 for 10 days. Once cells had cytopathic effect

(CPE), supernatants were gathered for serotype analysis. The cells were used for indirect immunofluorescent t assay.

3. Indirect immunofluorescent assay (IFA) [12]

Cells that had CPE were fixed on slide, and incubated with monoclonal mouse antibodies (CHEMICON Inc, CA, USA) against different enteroviruses. The cover slips were then incubated with FITC labeled anti-mouse sera after washing. Positive cells were identified as green cells under fluorescent microscope.

4. Nneutralization test [13]

Viral strains identified by IFA were inoculated into the cell lines used to isolate the viruses until significant CPE is present to increase the amount of viruses. Viral titers ($CCID_{50}$) were measured after freezing and thawing, and diluted to 100 $CCID_{50}$. 96-well plates were used and 20 u/50 ul of Anti-CA24 variant anti-sera (in house) was used for neutralization. CPE was monitored for 4 consecutive days to confirm the serotypes.

5. RNA extraction

Viral RNA purification kit (QIAGEN Inc, CA, USA) was used for RNA purification. 140 ul of samples were mixed with 560 ul Buffer AVL for 10 min at room temperature. 560 ul of absolute ethanol were then added and the samples were passed through QIAmp spin columns. The columns were washed with Buffer AW twice, and 80℃ RNase free water was used to elute the RNA. Viral RNA was used for reverse transcription polymerase chain reaction (RT-PCR)

6. Enterovirus real-time RT-PCR [14]

Primers and probes are designed at the 5'UTR highly conserved gene fragment of enteroviruses. Forward primer EV-F:

5’-CCCCTGAATGCGGCTAATC-3’ (position: 450-468); reverse primer (EV-R) 5’-GATTGTCACCATAAGCAGC-3’ (position: 580-5); probe EV-Probe: 5’-FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA-3’. ABI7900 was used with reaction condition 48℃ 30 minutes, 95℃ 10 minutes, cycles 95 ℃ 30 seconds, 60℃ 1 minute, 40 cycles.

7. CODEHOP reverse transcription- seminested PCR (RT-snPCR) [15]

5 ul of RNA was mixed with 5×PCR buffer, 10 uM primer mix (primers AN32, AN33, AN34, and AN35), 20 U RNase out, 100 uM dNTP, 0.01 M DTT, 100 U of SuperScript II reverse transcriptase (Invitrogen) and reacted at 22℃ for 10 minutes, 42℃ for 60 minutes, 95℃ for 5 minutes. 10 ul cDNA was used for PCR1 reaction with 2×PCR buffer, 200 uM dNTP, 50 pmol primers 224 and 222, 0.5 U Taq DNA polymerase (Invitrogen), and water to 50 ul. The PCR reaction condition was 95˚C for 30 seconds, 42˚C for 30 seconds, 60˚C for 45 seconds with a total of 40 cycles. The product is about 700 bp. For the second PCR reaction, 1ul PCR1 product was mixed with 40 pmol primers AN89 and AN88, 200 uM dNTP, 2.5 U Taq DNA polymerase (Invitrogen) with a total volume of 50 ul. The PCR condition was 95˚C for 30 seconds, 60˚C for 20 seconds, 72˚C for 15 seconds with a total of 40 cycles. The product is about 350 bp～400 bp.

8. Reverse transcription polymerase chain reaction (RT-PCR)

5 ul of RNA was used for RT-PCR. A total of 50 ul included SuperScript III RT (Invitrogen, USA) 200 U, Taq DNA polymerase (Invitrogen, USA) 5 U, 2×PCR buffer, 200 μM dNTP, 50 pmol primers to amplify 3C gene fragments. ABI 9700 PCR thermal cyclers were used for RT-PCR reaction: 42℃ for 50 minutes, 94℃ for 5 minutes, and 34 cycles of 94℃ for 60 seconds, 55℃ for 45 seconds, 72℃ for 60 seconds, and finally 72℃ for 10 minutes.

Primer：

D1 (forward primer)	ACAAACTGTTTGCTGGGCA	nt 5371to 5390
U2 (reverse primer)	ACTTCTTTTGATGGTCTCAT	nt 6025to 6044

9. Phylogenetic tree analysis

Molecular Evolutionary Genetics Analysis (MEGA) version 3.0 and "Neighbor-joining" method was used for phylogenetic tree analysis with bootstrap 1,000 times.

Results

Analysis of profiles of cases

The 91 cases in this study were from five geographic areas, including five cases from Wen-Shang District of Taipei City, 49 cases from Chi-Do, Chung-Cheng, An-Le, Chung-Shan, Ren-Ai, Sing-Yi, and Nuan-Nuan Districts of Keelung City, 15 cases from Shi-Jr City, Banchiao City, Sing- Chuang City of Taipei County, 14 cases from Do-Liu City, Ching-Tung, Shi-Lo, Hu-Wei of Yunlin County, and eight cases from Shing-Kang, Ya-Li, and Ming-Shiong of Chia-Yi County. Most of the cases were from Keelung City and the areas covered the whole city. The male to female ratio of cases was 1.1:1 and distributed across all age groups. Sixteen cases were aged <10 years, 24 cases were aged 11-20 years, nine cases were aged 21-30 years, 15 cases were aged 31-40 years, 15 cases were aged 41-50 years, and two cases were aged >50 years. The youngest case was two-month-old, and eldest was 86-years old (Table 1).

Virus isolation and identification

Among the 91 samples, 61 showed CPE in the Hep-2c cell line. Isolation rate was 67.0%. Overall, 93.4% of the positive samples showed CPE in four

days, with a average of three days. IFA showed that 60 positive cell samples were positive for coxsacikevirus A24, and one for Adenovirus.

Neutralization Test (serotype identification)

One viral strain was picked from each of the five geographic areas for neutralization test, and serotype identification and confirmation. All of the viral strains could be neutralized at $CCID_{50}$ by 20u / 50 ul of anti-CA24 variant anti-sera with a titer of 640~42000. Serotype was identified as CA24v.

Enterovirus Real-time RT-PCR

The 91 samples were used for RNA purification by viral RNA purification kit. Fifty-seven uncultured samples were positive for enterovirus, with a positive rate of 62.6%.

CODEHOP reverse transcription- seminested PCR

Ninety-one uncultured samples were centrifuged and tested with this method through PCR twice followed by electrophoresis. VP1 gene fragments 350-400 bp were amplified and sequenced. The sequences were analyzed by Nucleotide-nucleotide BLAST on the National Center of Biotechnology Information (NCBI) website. All the VP1 gene fragments amplified from the 72 samples in this outbreak belonged to CA24v, with a positive rate of 79.1% (Table 2).

RT-PCR

Sixteen viral strains were confirmed by IFA and neutralization test. Their 3C gene fragments were amplified, sequenced, and analyzed by Nucleotide-nucleotide BLAST on the National Center of Biotechnology Information (NCBI) website. The results showed that the fragments had highest similarity with CA24v.

Phylogenetic tree analysis

Forty-five viral strains, including 14 CA24v isolated in 2007, and reference and prototype strains isolated from different geographic areas were used to build phylogenic tree of the 3C gene fragments. The results showed that CA24v isolated in this outbreak belongs to cluster 6 of genotype III. Nucleic acid similarity between strains was 99.0-100%. They were also in the same cluster as Dso-26/2005 and Dso-52/2005 isolated in Singapore in 2005, with nucleic acid similarity of 97.8-98.6%. The outbreak in Taiwan in 1986 was cluster 1 of genotype III (AHC10-1), with nucleic acid similarity of 92.2-93.0% (Fig. 1).

Discussion

In this “pink eyes” outbreak, multiple methods, including molecular biology and traditional virology, were used to confirm the pathogen for early intervention of disease spread. Traditional cell culture was used for pathogen isolation, in addition to enterovirus real-time RT-PCR, CODEHOP reverse transcription- seminested PCR, and indirect immunostaining of centrifuged clinical samples. This is the first time that indirect immunostaining of centrifuged clinical samples was used. CPE is affected by multiple factors, including sample handling, shipping, processing before inoculation, and sensitivity of cell lines. These can affect the time to isolation, and hence CPE is not a timely method to identify virus for disease prevention. Although appearance of pathogen provides a more direct evidence compared to molecular biological tests, molecular biological tests are still more timely, and therefore, more feasible for disease control. Enterovirus Real-time RT-PCR was then used to confirm the existence of enterovirus, but not the serotypes. While we were

doing cell culture, centrifuged clinical samples with residual cells were used for fluorescent staining using commercial kits. Parts of the samples showed positive reactions toward pan-enterovirus and anti-CA24 monoclonal antibodies. Reaction toward anti-EV70 was negative. (By this time, the pathogen of the AHC outbreak is almost identified.) CODEHOP reverse transcription- seminested PCR was first applied to perform PCR directly using clinical samples for serotype identification. The amplified fragments could be sequenced, and showed high similarity to CA24. Now it is clear that the pathogen was enterovirus serotype CA24. Neutralization test, although it is time consuming, demanding, and requires different anti-sera, and hence cannot be used widely, it is still the gold standard for serotype identification and confirmation of enterovirus. We picked five strains isolated from different geographic areas, and confirm them to be coxsacikevirus A24 variant.

Taiwan is an island in the west pacific area. The sub-tropical climate and high population density is suitable for disease transmission. In 2007, Taiwan had another outbreak of AHC caused by CA24v. This incident showed the relationship between the characteristics of the pathogen, mode of transmission, age distribution, geographic differences, herd immunity, preventive measures, and molecular epidemiology. Since it took only three days to isolate the pathogens by cell culture, the viral loads were high and the proliferation was also quick, increasing the rate of transmission. This was the first significant AHC outbreak since 1994, and was the first that the disease originated from Keelung City of northern Taiwan, then disseminated. Herd immunity determines the amplitude of an outbreak. According to a study from Kaohsiung University on CA24v and EV70, before 1981, antibody toward CA24v was present in only 4.3% of the population, and EV70 in 34.0%, indicating that

although EV70 caused outbreaks in Taiwan in 1971, it had been transmitted before. Before 1985, CA24v infections only occurred sporadically, causing decreased herd immunity, and provided suitable condition for the next outbreak. CA24v belongs to Piconaviridae Enterovirus, but age distribution of CA24V infection patients and outbreaks were different from other enteroviruses in Taiwan. According to the CDC viral pathogens surveillance system, patients of enteroviruses are usually young, but CA24v occurred in persons of high activity ages. Persons aged <10 years consisted of the smallest age group of CA24v infections, reflecting the mode of transmission by life styles.

Molecular epidemiology analysis showed that CA24v was first isolated in 1970. The ancestor evolved with time and place. The evolution rate is about $3.7x10^{-3}$/year. On the 3C phylogenic tree, CA24v from 1970-2005 can be separate into three genotypes. The genotypes have temporal relationship with the year of isolation. In 1970-1971, it was genotype I, 1975, genotype II, and 1985-2005, genotype III. The genotype was transmitted worldwide, and separated into six subtypes (genotype III cluster 1-6) in different time and geographic areas. Each subgenotype was isolated from different outbreaks. Since CA24v appeared in Taiwan in 1985, and outbreaks of different magnitudes occurred in different times, all CA24v belonged to genotype III. The subgenostypes, however, were different. In 1985-1986, it was genotype III cluster I, 1988-1989, genotype III cluster 5, 2001-2002 and 2007, genotype III cluster 6. The whole genotype III cluster 6 (Fig. 1) can be separated into two lineages. The strains isolated in Taiwan in 2000-2001 were lineage 1, those isolated in 2002 and 2007 were lineage 2. As shown from the phylogenic tree, in 2002-2004, many areas had CA24v outbreaks belonging to genotype III cluster 6 lineage 2. Although strains from Singapore in 2005 and from Taiwan

in 2007 were all genotype III cluster 6 lineage 2, with changes in time and geographic area, genotype III cluster 6 can be separate into 3 lineages, and genotype III cluster 6 lineage 3 is strains isolated after 2005.

This AHC outbreak ended within a month, and the strategies for disease control was important. Frequent hand washing, stopping sharing towels, preventing symptomatic patients from entering public areas including schools and offices, and strengthening personal hygiene were all effective ways to stop or decrease pathogen transmission. This outbreak occurred in fall, when the activity of enterovirus is decreased compared with summer. According to molecular epidemiology, in southern Taiwan, between 1986 and 2003, strains were of the same lineages in two consecutive years. Hence reporting and monitoring of pink eyes should be emphasized to prevent another outbreak.

Several lessons learned from the AHC outbreak caused by CA24v in 2007 in Taiwan were:

1. The lab used CODEHOP RT sn-PCR for the first time and used residual cells from clinical samples for IFA for timely diagnosis.
2. This AHC outbreak was the first one in the Keelung City of the northern Taiwan since 1994, indicating that the pathogen is circulating in the environment, waiting for uninfected host. In addition, the relationship between herd immunity and susceptible hosts would lead to breakage of transmission or an outbreak.
3. Age distribution and time to isolate the pathogen reflected the mode and rate of transmission.
4. The outbreak ended within one month, showing that the mode of transmission between viruses and the importance of preventive strategy.

References

1. Kurokawa M, Rai SK, Ono K, et al. Viral investigation of acute hemorrhagic conjunctivitis outbreak (2003) in Nepal using molecular methods. Southeast Asian J Trop Med Public Health 2006;37:904-10.
2. Dussart P, Cartet C, Huguet P, et al. Outbreak of acute hemorrhagic conjunctivitis in French Guiana and West Indies caused by coxsackievirus A24 variant: Phylogenetic Analysis Reveals Asian Import. J Med Virol 2005;75:559-65.
3. Gopalkrishna V, Patil PR, Kolhapure RM, et al. Outbreak of acute hemorrhagic conjunctivitis in Maharashtra and Gujarat States of India, caused by coxsackie virus A-24 variant. J Med Virol 2007;79:748-53.
4. Yeo DS, Seah SG, Chew JS, et al. Molecular identification of coxsackievirus A24 variant, isolated from an outbreak of acute hemorrhagic conjunctivitis in Singapore in 2005. Arch Virol 2007,152: 2005-16.
5. Triki H, Rezig D, Bahri O, et al. Molecular characterisation of a coxsackievirus A24 that caused an outbreak of acute haemorrhagic conjunctivitis, Tunisia 2003. Clin Microbiol Infect 2007;13:176-82.
6. Lin KH, Wang HL, Sheu MM, et al. Molecular epidemiology of a variant of coxsackievirus A24 in Taiwan: two epidemics caused by phylogenetically distinct viruses from 1985 to 1989. J Clin Microbiol 1993;31:1160-6.
7. Chang CH, Lin KH, Sheu MM, et al. The change of etiological agents and clinical signs of epidemic viral conjunctivitis over an 18-year period in southern Taiwan. Graefes Arch Clin Exp Ophthalmol 2003;241:554-60.

8. Lin KH, Chern CL, Chu PY, et al. Genetic analysis of recent Taiwanese isolates of a variant of coxsackievirus A24. J Med Virol 2001;64:269-74.
9. Ishiko H, Takeda N, Miyamura K,et al. Phylogenetic analysis of a coxsackievirus CA24 varient: The most recent worldwide pandemic was caused by progenies of a virus prevalent around 1981 Virology 1992;187: 748-59.
10. Isenberg HD. Essential procedures for clinical microbiology, American Society for Microbiology, Washington, DC. 1998:451-523.
11. Park SW, Lee CS, Jang HC, et al. Rapid idetification of the coxsackievirus A24 variant by molecular serotyping in an outbreak of acute hemorrhagic conjunctivitis. J Clin Microbiol 2005;43:1069-71.
12. Rigonan AS, Mann L, Chonmaitree T. Use of monoclonal antibodies to identify serotype of enteroviurs isolate. J Microbiol 1998; 1877-81.
13. Schidt NJ and Lennette EH. Advances in the serodiagnosis of viral infections. Prog Med Virol 1973;15:244-308.
14. Lin TL, Wang SF, Yang JY, et al. Application of real-time RT-PCR in enteroviruse detection from clinical specimens. Epidemi Bullet 2005;21;436-50.
15. Nix WA, Oberste MS, Pallansch MA, et al. Sensitive. Seminested PCR amplification of VP1 sequences for direct idetification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol 2006;44:2698-704.
16. Thompson JD, Higgins DG, Gibson TJ.e al. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673-80.

17. Lin KH, Sheu MM, Huang WL, et al. Seroepidemiological study of coxsackievirus type A24 variant (CA24v) pre and post-epidemic in Taiwan.Kaohsiung J Sci 10:606-12

Table 1. Profiles of cases

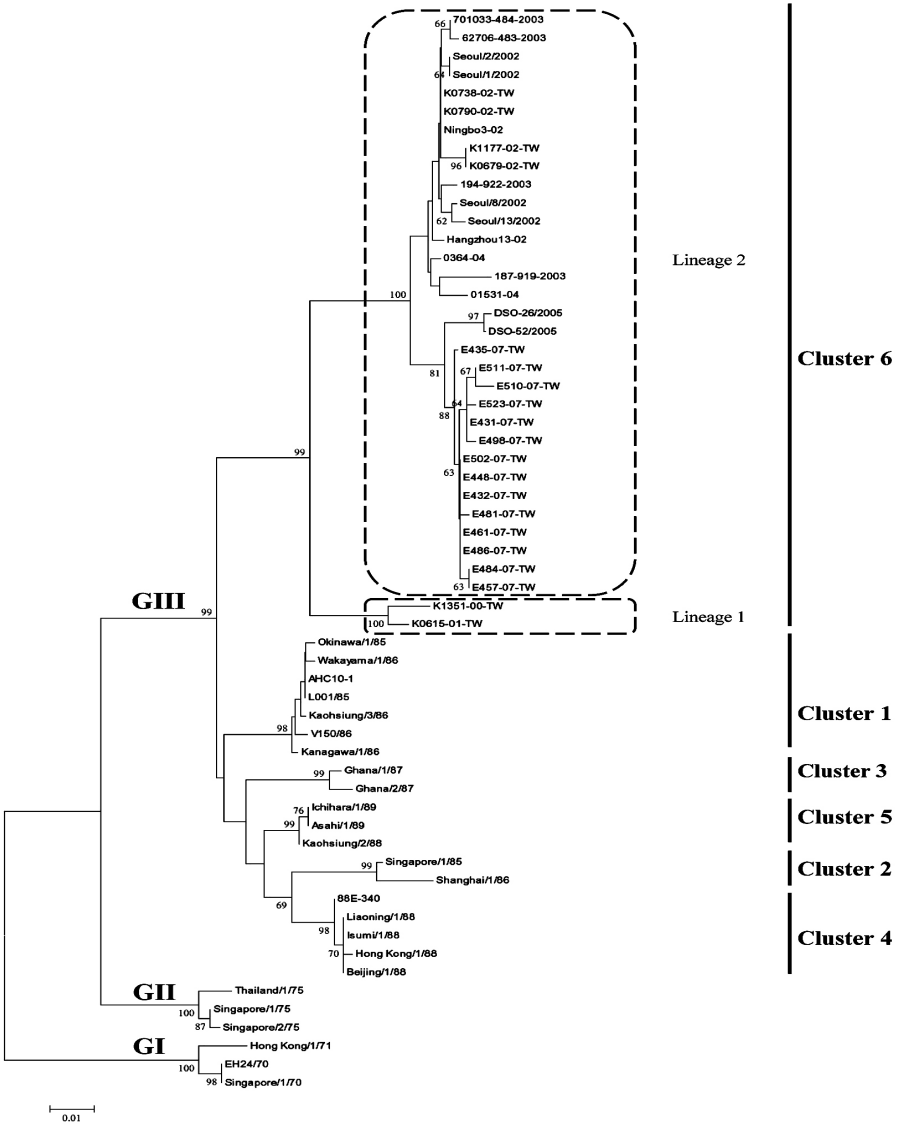
Areas	Number of cases	M:F	Age distribution (years)					
			<10	11-20	21-30	31-40	41-50	>50
Taipei City	5	1.5:1	0	3	1	1	0	0
Keelung City	49	1.45:1	10	14	3	8	10	4
Taipei County	15	1:1.5	1	3	4	3	3	1
Yulin County	14	1:1	4	1	0	1	1	7
Chia-Yi County	8	1:1	1	3	1	2	1	0
Total	91	1.1:1	16	24	9	15	15	12

Table 2. Results of different examination methods of conjunctival swabs

Cell culture	Number of Samples								
	CODEHOP RTsn-PCR			Real time PCR			Pellet IFA*		
	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative
Positive	61	57	4	61	51	10	35	12	23
Negative	30	15	15	30	6	24	5	0	5
Total	91	72	19	91	57	34	40	12	28

*IFA staining of pellet of conjunctival swabs after centrifuge

Figure 3. Phylogenic tree of the 3C region 511nt of CA24v between 1970 and 2007



"Neighbor-joining"method, Bootstrap 1,000 times