厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）

平成25年度　分担研究報告書

研究課題名：「アジアの感染症担当研究機関とのラボラトリーネットワークの促進と共同研究体制の強化に関する研究」

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「*V. cholerae* non-01/nonm-0139及び*V. fluvialis*の血清型別関する研究－中国」

研究要旨　　本研究は、わが国をはじめアジア各国で発生する細菌性下痢症に対応するため、主として食水系由来腸管感染症を対象にO血清型別ならびに病原因子の探索を行い、その発生と流行の傾向についての調査を主眼としている。本年度は中国 CDC（CCDC） の細菌部門とコンタクトを持ち、*V. cholerae* non-01/nonm-0139及び*V. fluvialis*の血清型別に関する共同作業を行った。

A. 研究目的

コレラ流行地においては、コレラ菌 *V. cholerae* 01, 0139 以外に *V. cholerae* non-01/non-0139(以下、ナグビブリオ)による下痢症も発生しており、また、その選択分離培地であるTCBS寒天培地に*V. cholerae*と同様の黄色集落を形成する*V. fluvialis*も検出される。コレラ流行地以外においても、ナグビブリオによるコレラ様の下痢症の報告もあり、O血清型別による発生の疫学解析が大いに有効であることが示されている。本研究はナグビブリオ及び*V. fluvialis*の血清型分布とその病原因子保有状況について、中国における状況調査を主たる目的とする。

B. 研究方法

中国においてヒト及び環境水から分離されたナグビブリオ及び*V. fluvialis*を中国側カウンターパートのDr. Biao Kanよりそれぞれ30株、10株分与してもらい、生化学性状試験と簡易同定キットEB20による菌種の同定、菌種特異的検出PCRにより各菌株の確認試験を行った。*V. cholerae* の病原因子の探索のため、*ctx*、*nag-st*、*hly*、T3SS、MARTX についてそれぞれの遺伝子検出PCRを行った。

C. 研究結果および考察

菌株リストは表1の通り。ナグビブリオは患者下痢便由来が10株、環境水由来が20株の計30株、*V. fluvialis*は患者下痢便由来が9株、魚由来株が1株であった。分離年はナグビブリオが2007～2010年、*V. fluvialis*が1963～2010年であった。

まず常法に従い、ビブリオ属菌の選択分離

培地である TCBS 寒天培地に接種したところ、約 1/4 が全く発育しないか、発育不良であった。特に *V. fluvialis* は 6 株(60%)が発育不良であった。それらの株は、非選択培地の TSA 培地に接種しなおし、全てが発育することを確認し、そこで発育したものを再度 TCBS 寒天培地に接種したが、やはり同様に発育不良であった。

それぞれの菌株を、TCBS 寒天培地で発育したものはそこから発育の良い黄色集落を、それ以外は TSA から任意の集落を 1 つ選択し、TSI 培地、LIM 培地、NaCl 濃度 0、0.1、3、8 % の Nutrient broth に接種し、また日水製薬の簡易同定キット EB20 にも接種し一夜培養した。

生化学的性状から得られた成績を表 1 に示した。その結果、ナグビブリオとして送付された VUN7 は *V. parahaemolyticus*、VUN21、22 は *Aeromonas sobria*、MJ-34、35 は *A. hydrophila* として同定された。*V. fluvialis* は 1 株を除き、*V. fluvialis* として同定された(後述)。

V. parahaemolyticus は極めて稀に白糖分解菌が分離される例があるが、VUN7 は白糖非分解菌であった。また、*Aeromonas* は白糖分解菌ではあるが、通常 TCBS 寒天培地には発育しない。実際 VUN21、22、 MJ-34、35 は、我々の試験では TCBS 寒天培地には発育しなかった。培地メーカによっては抑制力が弱く、*Aeromonas* 等が発育する場合もあるという。中国におけるこれらの培地や生化学的性状試験のやり方については、今後連絡を取り合って同定の精度管理として進めていくべきであると考えられた。

コレラの流行地においては、ナグビブリオによる下痢症も多発しており、また、それと同時に *V. fluvialis* による下痢症の発生も見られる。患者便からのコレラ菌の選択分離には通常 TCBS 寒天培地をもちいるため、白糖分解菌である両者を区別することは出来ない。そこで、迅速簡便に両者を区別する PCR をすでに開発してあったので、今夏にの分離株についてもこれを行ったところ、表 1 に示すようにナグビブリオのみ *ompW* 陽性で、*V. fluvialis* のみ *VftoxR* 陽性の成績が得られた。この PCR の系についても今後中国側に検討を依頼する予定である。

ナグビブリオに関しては、ヒトに対する病原因子の有無が下痢症との関連から重要な点である。表 2 に示すように、VUN3 は ctx 陽性で、コレラ毒素産生性であることが考えられる。また、ZJ201-1 は環境水からの分離菌であるが、nag-st 陽性であった。その他は、III 型分泌機構が VUN1 を除くすべての患者株で陽性であり、同様に MARTX も VUN1 を除くすべての患者株で陽性であった。環境水からの分離菌で III 型分泌機構や MARTX 陽性の株はヒトに対する病原株である可能性が高いことが推測される。

生化学的性状がオキシダーゼを除き *V. fluvialis* に一致する 1 株(JS2)と TCBS 寒天培地に発育しなかった株(VF12)をハウスキーピング遺伝子である gyrB の塩基配列について、参照株やデータベース上の他の菌種との比較を行った。図 1 に示すように、JS2(93gyrB)、VF12(92gyrB) は参照株の

VflugyrBとほとんど同一の配列であり、オキシダーゼ陰性のVibrioである*V. metschnikovii*とは異なることが明らかになり、JS2(93gyrB)、VF12(92gyrB)は*V. fluvialis*と同定された。

D. 結論

今回中国との共同研究において、中国での分離株を日本側に分与してもらうことが出来、共同研究の基本となる材料の共有ができたことは、今後の共同研究を行う上でも非常に重要な点である。

菌種の同定に関して一部問題が見られるが、情報の共有を行うことや技術の支援を行うことでこれからさらに発展が見込まれるものと期待される。

中国側からは*V. fluvialis*の血清型について、非常に興味を持って取り組みたいと申し入れがあり、今後さらに発展させていくことが両国ラボラトリーの共通認識である。

E. 健康危機情報

特になし

F. 研究発表

特になし

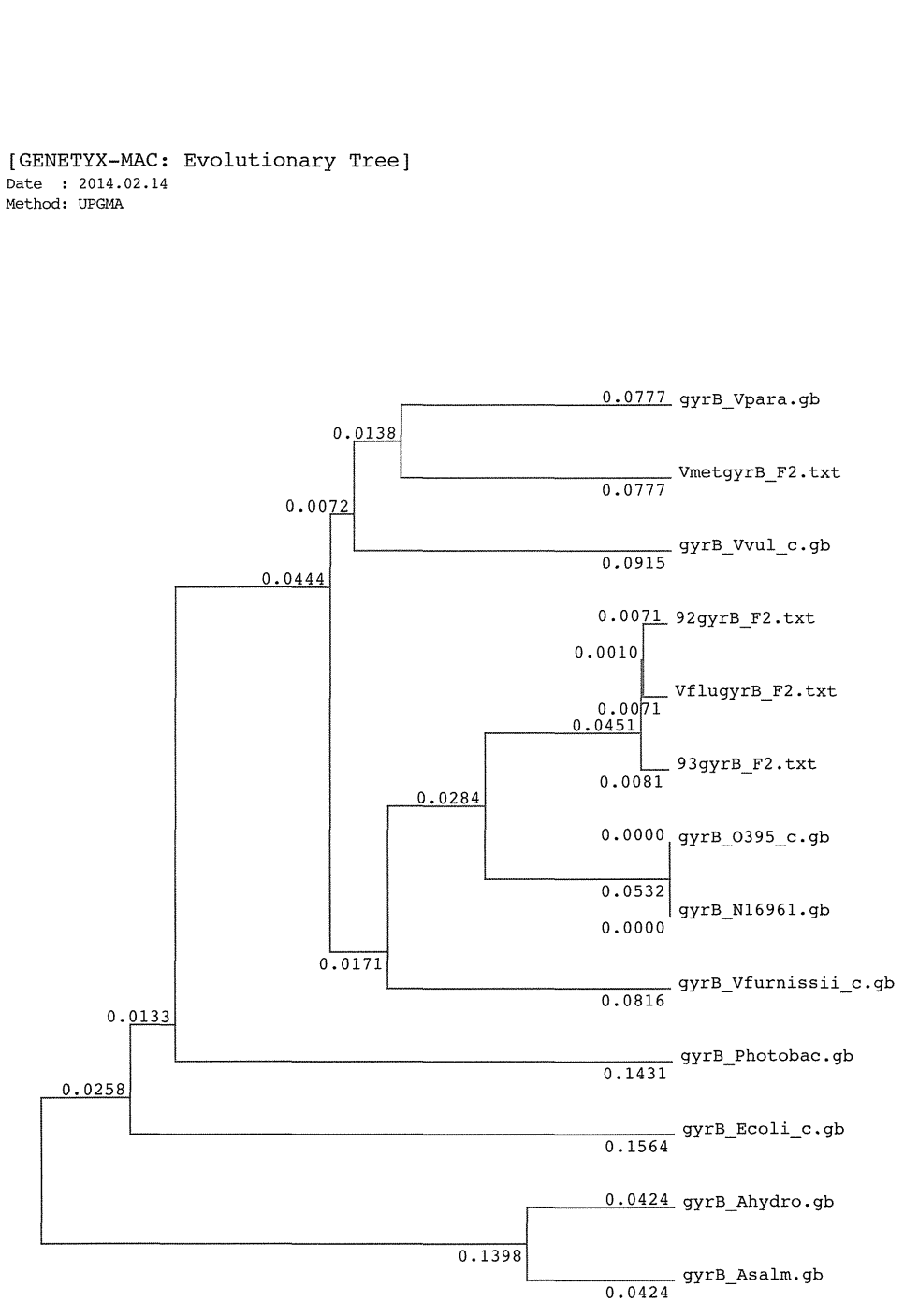

図 1　オキシダーゼ陰性の *V. fluvialis* の *gyrB* シークエンスによる解析。

92gyrB: TCBS で発育しない *V. fluvialis*、93gyrB: オキシダーゼ陰性の *V. fluvialis*、VflugrrB: *V. fluvialis* 参照株、VmetgyrB: *V. metschnikovii* 参照株、gyrB_: データベースに登録されている *gyrB* 遺伝子(Vpara: *V. parahaemolyticus*, Vvul: *V. vulnificus*, O395: *V. cholerae* O395, N16961: *V. cholerae* N16961, Vfurnissii: *V. furnissii*, Photobac: *Photobacterium damsela*, Ecoli: *E. coli,* Ahydro: *A. hydrophila*, Asalm: *A. salmonisida*

表1　中国CDCの分離株

Strain ID	Province/Country	Year isolated	Source	Serogroup	Species	ompW	VftoxR
VUN1	Xinjiang/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	+	-
VUN2	Xinjiang/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	+	-
VUN3	Sichuan/China	2008	Stool	non-O1/O139 V.cholerae	V. cholerae	+	-
VUN5	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	+	-
VUN6	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	+	-
VUN7	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. parahaemolyticus	-	-
VUN8	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	+	-
VUN9	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	+	-
VUN21	Hainan/China	2008	Stool	non-O1/O139 V.cholerae	A. sobria	-	-
VUN22	Hainan/China	2008	Stool	non-O1/O139 V.cholerae	A. sobria	-	-
MJ-33	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
MJ-34	Fujian/China	2007	Water	non-O1/O139 V.cholerae	A. hydrophila	-	-
MJ-35	Fujian/China	2007	Water	non-O1/O139 V.cholerae	A. hydrophila	-	-
MJ-36	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
MJ-37	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
MJ-38	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
MJ-39	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
MJ-40	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
MJ-41	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
MJ-42	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ193-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ194-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ194-3	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ195-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ196-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ197-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ198-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ199-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ200-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
ZJ201-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	+	-
85-142	Fujian/China	1985	Fish	V. fluvialis	V. fluvialis	-	+
63112	Guangdong/China	1963	Stool	V. fluvialis	V. fluvialis	-	+
VF2	Fujian/China	1985	Stool	V. fluvialis	V. fluvialis	-	+
VF12	Fujian/China	1985	Stool	V. fluvialis	V. fluvialis	-	+
JS2	Jiangsu/China	1987	Stool	V. fluvialis	V. fluvialis	-	+
JS54	Jiangsu/China	1987	Stool	V. fluvialis	V. fluvialis	-	+
EF85001	Xinjiang/China	1985	Stool	V. fluvialis	V. fluvialis	-	+
EF85002	Xinjiang/China	1985	Stool	V. fluvialis	V. fluvialis	-	+
liao85-50	Liaoning/China	1984	Stool	V. fluvialis	V. fluvialis	-	+
Ma2598	Anhui/China	2010	Stool	V. fluvialis	V. fluvialis	-	+

表2 ナグビブリオのPCRによる病原因子検索

Strain ID	Province/Country	Year isolated	Source	Serogroup	Species	ctx	nag-st	hly	T3SS(vscV2)	MARTX
VUN1	Xinjiang/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
VUN2	Xinjiang/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	-	-	+	+	+
VUN3	Sichuan/China	2008	Stool	non-O1/O139 V.cholerae	V. cholerae	+	-	+	-	+
VUN5	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	-	-	+	+	+
VUN6	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	-	-	+	+	+
VUN7	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. parahaemolyticus	-	-	-	+	-
VUN8	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	-	-	+	+	+
VUN9	Anhui/China	2010	Stool	non-O1/O139 V.cholerae	V. cholerae	-	-	+	+	+
VUN21	Hainan/China	2008	Stool	non-O1/O139 V.cholerae	A. sobria	-	-	-	-	-
VUN22	Hainan/China	2008	Stool	non-O1/O139 V.cholerae	A. sobria	-	-	-	-	-
MJ-33	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	+
MJ-34	Fujian/China	2007	Water	non-O1/O139 V.cholerae	A. hydrophila	-	-	-	-	-
MJ-35	Fujian/China	2007	Water	non-O1/O139 V.cholerae	A. hydrophila	-	-	-	-	-
MJ-36	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	+
MJ-37	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
MJ-38	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	+
MJ-39	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	+
MJ-40	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
MJ-41	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
MJ-42	Fujian/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
ZJ193-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
ZJ194-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
ZJ194-3	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
ZJ195-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
ZJ196-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	-
ZJ197-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	+	+
ZJ198-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	+	+
ZJ199-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	;	+	+
ZJ200-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	-	+	-	+
ZJ201-1	Guangdong/China	2007	Water	non-O1/O139 V.cholerae	V. cholerae	-	+	+	-	+

Report 2013

STUDY GENERAL TITLE

Laboratory-based collaboration net work of infectious diseases in Asia

PI general from CCDC: Xiao-Ping Dong, PhD & MD

Prof., Director of Division of Science and Technology, China CDC; Deputy Director of State Key Laboratory for Infectious Disease Control and Prevention, China CDC; Director, Dept. of Prion disease, National Institute for Viral Disease Control and Prevention, China CDC

1. Epidemiology of HFMD and genetic characterization of HEV71and CVA16

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1. Epidemiology

1.1. Profile of the outbreak in 2013

Totally, 1,828,377 cases were reported throughout year 2013, including 252 fatal cases. The number of reporting cases in year 2013 decreased compared with that in 2012 (2,170,248), and increased compared with that in 2011 (1,620,430). However, the number of fatal cases decreased dramatically, which is 509 in 2011, and 563 in 2012.

1.2. Age distribution

Cases were reported among all of the age groups, ranging from 0 to 85 years old. Young children less than 6 years old are still the majority suffering from HFMD, which account for 95% of the reporting cases. Young children especially 1-year-old group indicated highest incidence and fatality.

1.3. Spatial distribution

In 2013, there were cases reported from all over China. The top 6 provinces of reporting cases are Guangdong, Guangxi, Zhejiang, Hunan, Jiangsu, and Anhui, reporting cases of which account for 53% of national reporting cases. By fatal cases, Sichuan, Anhui, Hebei, Chongqing, Hunan, and Yunnan were the top 6 provinces. By the incidence, Hainan was still the top 1, followed by Guangxi, Guangdong, Fujian, Zhejiang, and Shanghai provinces.

1.4. Week distribution (Figure 1)

HFMD cases were reported throughout the year in 2013. The case number began to increase from week 8, and reached the peak in week 22. The first, also

the largest, round epidemics were lasting from week 10-34, followed by the second round epidemics in autumn, from week 13-33. The amount of cases and fatalities reported during the first round epidemic account for 61% and 72% of the cases in 2013 respectively. The second epidemic peak occurred from week 36-41, including 13% reporting cases and 7% fatalities.

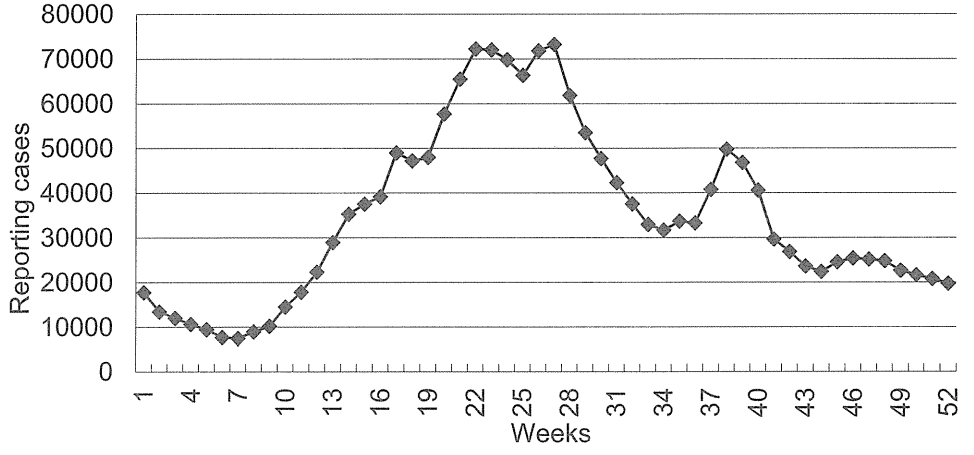

Figure 1. The reporting cases by week from Jan 1, 2013-Dec, 31, 2013

2. Pathogenic surveillance

2.1. Pathogenic spectrum (Figure 2)

In 2013, totally 87,454 cases, 4.8% of the reporting clinical cases, were confirmed by lab. The lab result indicated that other EV and EV71became the most major pathogen of HFMD in China, account for 48% and 37% of the positive respectively. EV71 is the dominant serotype during week 12-22. From week 22-49, other EV became the majority instead of EV71.

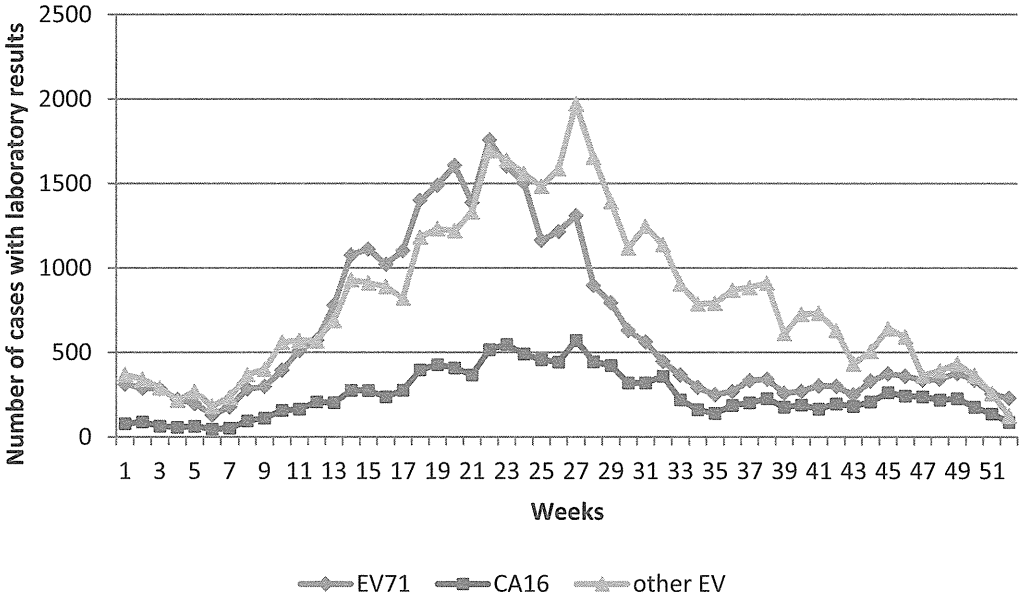

Figure 2. Pathogenic results in year 2013

2.2. Molecular epidemiology of EV71 and CA16

Phylogenetic trees of the EV71 and CA16 isolates in 2013 were reconstructed respectively, based on VP1 encoding region (891bp), with Kimura 2-parameter nucleotide substitution model. All the EV71 isolates in 2013 clustered together, showing the nearest phylogenetic relationship with subgenotype C4a, which was predominant in mainland China since 2004. For CA16, clade B1a and B1b still co-circulated in China in 2013. No predominance was found in specific provinces for both B1a and B1b.

2.3. Genomic characteristics

To clarify the genetic characteristics and the epidemic patterns of CVA16 in mainland China, comprehensive bioinformatics analyses were performed by using 35 CVA16 whole genome sequences from 1998 to 2011, 593 complete CVA16 VP1 sequences from 1981 to 2011, and prototype strains of human enterovirus species A (EV-A). Analysis based on complete VP1 sequences revealed that clade B1a and B1b were prevalent strains and have been co-circulating in many Asian countries since 2000, especially in mainland China for at least 13 years. While the prevalence of clade B1c (totally 20 strains) was much limited, only found in Malaysia from 2005 to 2007 and in France in 2010.

Genotype B2 only caused epidemic in Japan and Malaysia from 1981 to 2000. Both clade B1a and B1b were potential recombinant viruses containing sequences from other EV-A donors in the 5'-untranslated region and P2, P3 non-structural protein encoding regions.

1. Epidemiology of HFMD and genetic characterization of HEV71and CVA16

Study faculty: Institute for Viral Disease Control and Prevention, China CDC, Changbai road 155, Changping Qu, Beijing 102206, China

Name and official title of PI: Wenbo Xu, Prof., Assistant Director of National Institute for Viral Disease Control and Prevention in China CDC

3. Epidemiology

3.1. Profile of the outbreak in 2012

The nationwide epidemics of HFMD were continuing in China in 2012. Totally, 2,170,248 cases were reported throughout the whole year, including 20,949 severe cases, and 563 fatal cases (Table 1). The case number of all three types increased compared with that in 2011. However, the fatality rate were decreasing year by years, as showed in table 1.

Table 1. Summary of the outbreak during 2011-2012.

Items	2011	2012	2011 vs 2010	2012 vs 2011
Reporting cases	1,620,430	2,170,248	-9%	+34%
Severe cases	18,717	20,949	-33%	+12%
Fatal cases	509	563	-44%	+11%

3.2. Age distribution

Cases were reported among all of the age groups, ranging from 0 to 85 years old, but ~94% of the cases were young children less than 5 years old, which is consistent with the previous data in China. Furthermore, the amount of both reporting cases and fatal cases were indicated to be the largest in 1-year-old group, accounting for ~30% and ~47% of the all reporting cases and the fatal cases respectively. Young children especially 1-year-old group was showed to have higher risk for HFMD.

3.3. Spatial distribution

In 2012, the southeast of mainland China was still the most severe area for HFMD epidemic. By both the reporting cases and fatal cases, Guangdong, Guangxi, Hunan, Zhejiang, and Jiangsu were the top 5 provinces. However, by the incidence, Hainan was the top 1, followed by Guangxi, Guangdong, Hunan, and Zhejiang provinces.

3.4. Temporal distribution (figure 2)

HFMD cases were reported throughout the year in 2012. The case number began to increase from week ~10, and reached the peak in week ~21. The first, also the largest, round epidemics were lasting from week 10-34, followed by the second round epidemics in autumn, from week 35-50. The amount of cases and fatalities reported during the first round epidemic account for ~50% and 53% of the cases in 2012 respectively.

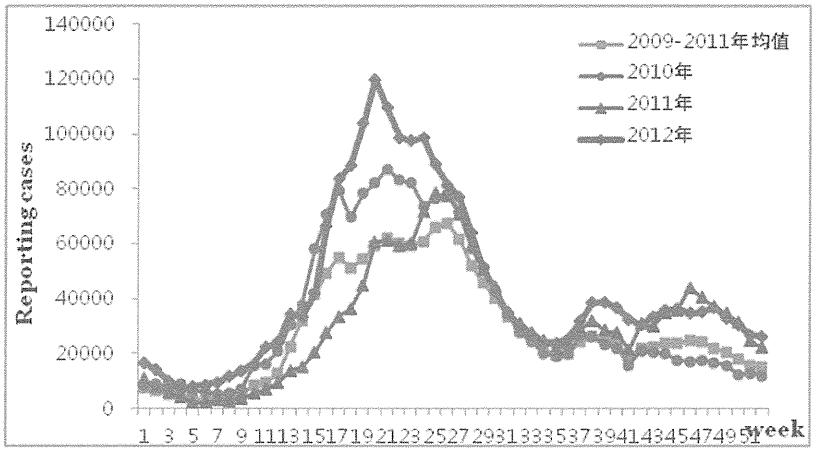

Figure 2. The reporting cases by week from Jan 1, 2010-Dec, 31, 2012

4. Pathogenic surveillance

4.1. Pathogenic spectrum

In 2012, totally 105,380 cases, 4.9% of the clinically reporting cases, were confirmed by lab. The lab result indicated that EV71 and CA16 still were the most major pathogen of HFMD in China, account for 48.9% and 30.6% of all of the positive respectively. Still there were 20.5% of the confirmed cases associated with other enteroviruses.

4.2. Molecular epidemiology of EV71 and CA16

Phylogenetic trees of the EV71 and CA16 isolates in 2012 were reconstructed respectively, based on VP1 encoding region (891bp), with Kimura 2-parameter nucleotide substitution model. All the EV71 isolates in 2012 clustered together, showing the nearest phylogenetic relationship with subgenotype C4a, which was predominant in mainland China since 1998. Different with the unique genotype of

EV71 circulating in China, there were 2 subgenotype of CA16, B1a and B1b, co-circulating in China in the recent years. No significant predominance were found in specific provinces for both B1a and B1b.

4.3. Genomic characteristics

16 complete genome sequences of EV71 were determined, isolated from HFMD patients during the large scale outbreak and non-outbreak years since 1998 in China. These 16 full length genome sequences were aligned with another 104 genome sequences of EV71 from China mainland, available in GenBank, covering the time period of 1998-2011. Our comprehensive recombination analysis showed the evidence of genome recombination of subgenotype C4 (including C4a and C4b) sequences between structural genes from genotype C EV71 and non-structural genes from the prototype strains of CAV16, 14 and 4, but the evidence of intratypic recombination between C4 strains and B subgenotype was not enough strong (Figure 3). This intertypic recombination of C4 viruses were first identified in 1998 and became the predominant endemic viruses circulating in China mainland for at least 14 years.

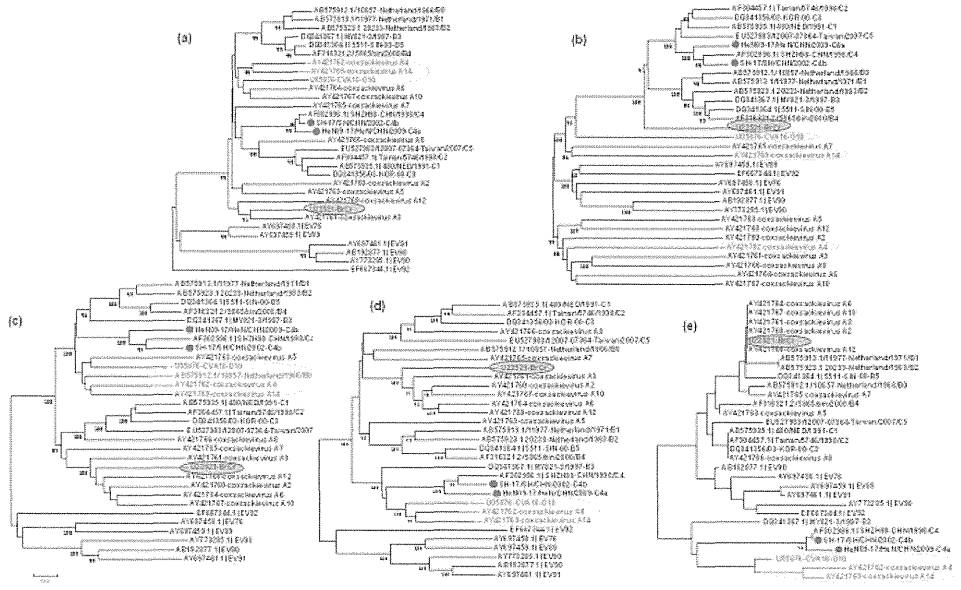

Figure 3. **Rooted phylogenetic trees showing the relationships amongst HEV-A isolates using the different genomic regions.** The neighbour-joining trees were constructed from alignment of the 5' UTR (a), P1 (b), P2 (c), P3 (d) and 3′UTR (e)

genomic region, respectively

8 complete genome sequences of CA16 from China were determined. Comprehensive phylogenetic and recombination analyses were performed on these 8 complete genome sequences and another 20 CA16 whole genome sequences from China in GenBank, during 1998 to 2011. Analysis indicated that CA16 B1a and B1b strains were potential multiple-recombinant viruses containing other HEV-A donor sequences in the 5'-untranslated region and P2, P3 non-structural protein encoding regions. The donor squences were hard to determined.

2. Laboratory based surveillance and outbreak detection for multiple foodborne diseases

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Summary:

A survey of etiology from the diarrheal patients was conducted in the sentinel city, Yuxi of Yunnan province. The stool samples were obtained from the hospitals and isolation of multiple target bacteria pathogens was conducted in Yuxi city CDC laboratory. Molecular subtyping of the isolates was also performed. Based on the one-year survey, the distribution of bacterial pathogens was obtained, diarrheagenic *Escherichia coli* (DEC), *Aeromonas* and *Salmonella* strains were listed in the top three bacteria species. PFGE of the isolates showed complex patterns, but some clusters with the same patterns were also found. A protocol of multiple bacteria isolation from the diarrheal sample was developed generated, which can be provided to the national laboratory surveillance network as the technical support in the foodborne disease survey. In another part of work, non-O1/non-O139 *V. cholerae* strains from the environmental samples and diarrheal patients in the previous surveillance, and the *V. fluvialis* strains form diarrheal patients were transferred to NIID for the serotype identification and further studies.

I. Purpose:

To establish the multiple foodborne pathogen detection protocol and molecular subtyping surveillance which can be used in the city and provincial health institutions, to strengthen the ability of cluster and outbreak detection through laboratory based surveillance. The distribution of the bacterial pathogens will be surveyed in the outpatients with the acute diarrhea in the sentinel city. In addition, an optimized

protocol for the multiple bacterial pathogen isolation from stool sample and a multiplex real-time PCRs detection protocol will be established for the surveillance of the common foodborne pathogens, to recommend to the network of PulseNet China. This year the study will focus on the laboratory based detection of the bacterial pathogens from the diarrheal cases, and molecular subtyping of the isolates, to find the possible cluster of the isolates. Yuxi city, in Yunnan province, is selected as the sentinel site. The study includes two parts: (1) Optimization of a protocol for the isolation of multiple bacterial pathogens from diarrheal cases in the city-level laboratory. The etiology of acute diarrhea using a sentinel hospital-based surveillance will also be analyzed. (2) Molecular subtyping (pulsed-field gel electrophoresis, PFGE) of the isolates in provincial CDC laboratory, to find the pattern cluster.

II. Methods:

Design of the project. The study focused on the pathogen isolation from the diarrheal patients in the health institution in the city level, which is an important step to connect the outpatients and molecular subtyping based surveillance in the network such as PulseNet. For this purpose, work plan of the project was made in Yuxi city, a district in Yunnan province. Four sentinel hospitals, including one children hospital, were selected. During the study, other three hospitals including one children hospital also joined and submitted some samples of the diarrheal outpatients. The samples were collected from the hospitals and transferred (in C-B transport media) to the laboratory in Yuxi Center for Disease Control and Prevention. A group from the Yunnan provincial CDC joined to coordinate the project in the field and worked in the laboratory simultaneously. The study conducted for one year, from Jun 2012 to May 2013.

Detection of bacteria from the outpatient samples. The fecal or swab samples of the outpatients with diarrhea were collected for the detection. The following bacteria were included as the targets for the isolation: *Salmonella*, *Shigella*, *diarrheagenic Escherichia coli* (DEC), *Vibrio*, *Plesiomonas shigelloides* and *Aeromonas*. The isolation methods for the different pathogens were integrated into a protocol,

including the enrichment or plating directly (for the isolation of *Shigella* and DEC), the suspected colony selection. The biochemical tests were used for the primary identification of the strains. For the DEC, the toxigenic related genes were detected with PCR for its identification. The biotypes and serotypes were determined with the corresponding methods and typing sera.

Pulsed-field gel electrophoresis (PFGE). These strains were analyzed by PFGE according to the protocols from PulseNet International in the laboratories of Yunnan CDC and China CDC.

III. Results:

1) Samples collected in the hospitals.

From Jun 1, 2012 to May 31, 2013, totally 1139 samples of diarrheal outpatients were collected, including feces and swabs. The samples were collected and cultured weekly for the isolation of the target bacteria. All ages of patients were covered. The samples were preserved in the C-B transport media and transferred to the CDC laboratory. The main symptoms of the patients were also recorded, most patients (53.2%) had 3-5 times of diarrhea per day, 42.3% had 6-10 times per day, and 4.5% had more than 10 times diarrhea per day. Other symptoms were also recorded.

Table 1. The collected samples and the isolation rates.

Hospital	Number of samples	Number of positive samples*	Positive rate (%)
Beicheng Central Hospital	303	77	25.4
Chunhe Hospital	191	48	25.1
Dayingjie Hospital	303	72	23.8
Liqi Hospital	23	8	34.8
Beiyuan Hospital	13	4	30.8
Yuxi Children Hospital	217	19	8.8
Yuxi Women and Children Health Care Hospital	89	9	10.1
Sum	1139	237	20.8

* The samples from which the target bacteria were obtained.

2) Isolation of the bacteria from the samples

In the 1139 samples, the target bacteria were recovered from 237 patients (20.8% positive rate), including 24 possible mixed infections, since more than one target bacteria species were obtained (Table 2). Mixed infection often occurred among DEC, *Aeromonas*, *P. shigelloides* and *Salmonella*, and didn't find *Shigella* and *Vibrio* involved (Table 3). Within all the 265 isolates, the distribution of bacteria from high frequency to low frequency is DEC (obtained from 10.45% patients), *Aeromonas* (7.37% in total), *Salmonella*, *P. shigelloides*, *Shigella*, and *Vibrio* (Table 4).

Table 2. Bacterial isolation in the samples of diarrheal patients.

Recovered bacterium	Number of samples	Recovery rate (%)
DEC	96	
Aeromonas	65	
Salmonella	21	
P. shigelloides	14	
Shigella	15	
V. paraheamolyticus	1	
V. fluvialis	1	
Mixed bacteria*	24	2.1
Sum	237	20.8

* More than one target bacteria were obtained from one sample.

Table 3. Mixed infection in samples.

Recovered bacterium	Number of samples
DEC and DEC	6
DEC and *A.hydrophila*	3
DEC and *P. shigelloides*	3
DEC and *A. sobria*	2
DEC and *Salmonella*	1
A.hydrophila and *A. sobria*	3
A.hydrophila and *P. shigelloides*	2
Salmonella, *P. shigelloides* and *A. sobria*	1
Salmonella, *A.hydrophila* and *A. sobria*	1
A.hydrophila, DEC and *P. shigelloides*	1
A.hydrophila, DEC and *A. sobria*	1

Sum	24

Table 4. The bacteria isolated from the diarrheal patient samples.

Bacterium	Number of isolates	Recovery rate (%)	Constituent ratio (%)
DEC	119	10.45	44.9
Aeromonas	84	7.37	31.7
Salmonella	24	2.11	9.1
P. shigelloides	21	1.84	7.9
Shigella	15	1.32	5.7
V. paraheamolyticus	1	0.09	0.4
V. fluvialis	1	0.09	0.4
Sum	265	23.27	100

3) The distribution of detection rate

During the period from 2012 June to 2013 May, the monthly detection rate of target bacteria was between 8% and 38.9% (Fig.1). The detection rate rose rapidly in February, reached the peak in March, began to decline after July, fell to the lowest value in January. The number of diarrhea patients had two peaks during the year, one in the summer with a high detection rate, and the other in the winter with a low detection rate.

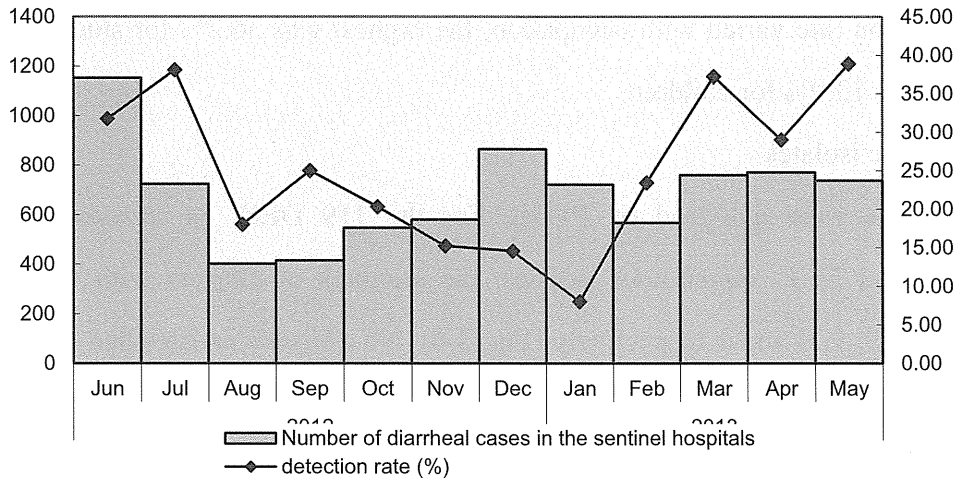

Figure 1. The monthly detection rate of target bacteria.

The target bacteria were isolated in every age group (Fig.2). For children below 5 years old, the detection rate was low and no *P. shigelloides* was isolated. For age