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Metaviromics Study on Populations in Areas Affected by Unexplained Sudden Death in Yunnan Province

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Abstract: *Objective*: To investigate the correlation between the occurrences of unexplained sudden death in Yunnan Province and the pathogen spectrum by using real-time fluorescent quantitative reverse transcription PCR, metagenomic next-generation sequencing (mNGS), and virus isolation techniques to test autopsy samples from cases of unexplained sudden death and fecal specimens from populations in affected areas. *Methods*: Real-time fluorescent quantitative reverse transcription PCR and Sanger sequencing were performed on 101 fecal samples collected from populations in affected areas. Virus isolation was conducted on fecal and gastric content samples from individuals who died suddenly. Additionally, metavirome sequencing and pathogen spectrum abundance detection were performed on 50 autopsy organ samples. *Results*: No specific fragments of enteroviruses were detected in 101 fecal samples from the population in the affected wards, and no viruses were isolated from fecal and gastric content samples of sudden death victims. Among the 50 autopsy organ samples, 29 were successfully sequenced. High-throughput sequencing revealed low-abundance enterovirus reads in 11 samples (relative abundance $\leqslant$ 0.91% in all cases); Enterovirus A114 was detected in 6 samples (with relative abundances of 0.211%, 0.571%, 0.910%, 0.013%, 0.002%, and 0.0000263%, respectively); Coxsackievirus A2 in 9 samples (with relative abundances of 0.111%, 0.192%, 0.051%, 0.291%, 0.007%, 0.00019%, 0.00342%, 0.000551%, and 0.0000368%, respectively); and Coxsackievirus B3 in 9 samples (with relative abundances of 0.312%, 0.486%, 0.120%, 0.765%, 0.001%, 0.001%, 0.001%, 0.0000999%, and 0.00000848%, respectively). Coexistence of 2-3 types of enteroviruses was observed in some samples. Genomic annotation results indicated that high-abundance bacteria were primarily *Paeniclostridium sordellii* and *Escherichia coli*, while viral species could not be successfully assembled due to their low abundance. *Conclusion*: Enterovirus infection may be one of the causes of some unexplained sudden

deaths in Yunnan, and the possibility of varying degrees of enterovirus infection cannot be ruled out in some populations in the affected areas. The detection of bacteria may be attributed to the normal intestinal flora of the human body or contamination during the autopsy sampling process.

Keywords: Heart; Unexplained sudden death in Yunnan; Autopsy specimens; Feces; Metagenomic next-generation sequencing; Enterovirus

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1. Introduction

The etiology of unexplained sudden death in Yunnan (referred to as "Yunnan sudden death") is highly complex and may result from the combined effects of multiple factors. Over four decades of researches have indicated that enterovirus infection could be one of the causes contributing to some cases of Yunnan sudden death [1]. Given the challenges in specimen collection and preservation due to the remote and mountainous locations of sudden death outbreak areas, coupled with the low sensitivity of etiological diagnostic methods at the time for detecting viral genomes in heart samples, a lack of knowledge regarding mutant viruses and "new" viruses associated with inflammatory cardiomyopathy, and unclear genetic backgrounds of families affected by sudden death, in-depth etiological diagnostic studies on some sudden death incidents have been hindered. To further explore and verify the association between the occurrences of Yunnan sudden death and pathogen infections, this study employed metagenomic viromics, real-time fluorescent quantitative reverse transcription PCR, and traditional virus isolation methods to test 50 autopsy organ samples from nearly 20 cases of Yunnan sudden death and 101 fecal samples from the population in the affected areas. The aim is to further investigate the etiology of Yunnan sudden death and provide guidance and basis for future prevention, control, and intervention efforts.

2. Materials and methods

2.1. Sample sources

A total of 101 fecal samples were collected from individuals in Yunnan's sudden death-affected areas between 2002 and 2025. Autopsies were performed on 20 sudden death cases, yielding 50 samples including tissues, organs, blood, feces, and gastric contents. The 101 fecal samples originated from sudden death cases, their family members, and residents of the same villages. The 20 sudden death cases were from 14 newly reported outbreaks between 2002 and 2020, distributed across four prefectures (8 in Dali, 9 in Chuxiong, 2 in Lincang, and 1 in Baoshan), 11 townships, and 14 natural villages in the province. Among these, 9 natural villages were previously identified as affected areas. The altitudes of the natural villages were as follows: 1 below 1,500 meters, 8 between 1,500 and 2,000 meters, and 5 above 2,000 meters. Geographically, these villages are located between longitudes 98°40'42.14" to 102°17'34.48" east and latitudes 24°51'03.276" to 26°29'49.99" north, all situated in mountainous or semi-mountainous regions. Among the sudden death cases, there were 13 females and 7 males. In terms of age distribution, there was 1 case aged 5 years, 1 aged 18 years, 6 aged between 19 and 30 years, 5 aged between 30 and 40 years, 5 aged between 40 and 50 years, and 2 aged over 50 years. Young

and middle-aged adults (aged 20–45 years) accounted for 65% (13/20) of the cases. Ethnically, 60% (12/20) were Yi, 30% (6/20) were Lisu, and the rest were Han. 75% (15/20) of the sudden death cases had relatives who were also affected by the same illness or had died suddenly (including a mother-daughter pair, LM and LXL), indicating a familial clustering characteristic. Samplings were conducted in accordance with the relevant requirements of the "Guidelines for the Prevention and Control of Unexplained Sudden Death in Yunnan" and the "Emergency Response Plan for Mass Unexplained Diseases (Trial)". The autopsies were performed by forensic pathologists or qualified professional technicians from hospitals at or above the county level, with informed consent forms signed by either the individuals themselves or the deceased's' family members. This study and all research methods employed have received approval from the Medical Ethics Committee of the Yunnan Provincial Institute for Endemic Disease Control and Prevention (YDES Medical Ethics [2017] No. 003) and the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention.

2.2. Research methods

Tissue, organ, and fecal samples were transported at low temperatures in October 2021 to the National Poliomyelitis Laboratory of the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, for relevant testing. Specifically, 101 fecal samples collected from individuals in the affected areas underwent real-time fluorescent quantitative reverse transcription PCR and Sanger sequencing. Fecal and gastric content samples from individuals who experienced sudden death were preprocessed according to standardized procedures, and the processed specimens were inoculated onto Human rhabdomyosarcoma (RD) cells to observe for cytopathic effect (CPE). Additionally, nucleic acids were extracted from 50 tissue and organ samples obtained from 20 cases of sudden death in Yunnan, followed by library construction, high-throughput sequencing, and bioinformatics analysis to describe the microbial profile and its abundance.

2.3. Main reagents and instruments

2.3.1. Reagents

Tianlong pre-packaged nucleic acid extraction kit (Ex-DNA/RNA virus (CDC)/T327), QIAamp Viral RNA Mini Kit (Qiag) for nucleic acid extraction, RNAsimple Total RNA Extraction Kit (DP419), PrimeScript™ One Step RT-PCR Kit Ver.2 (Dye Plus, RR057A), One Step PrimeScript™ RT-PCR Kit (064A) for fluorescent quantitative reverse transcription PCR, MGIEasy-rRNA Removal Kit (1000005953), MGIEasy RNA Library Preparation Kit (1000005276), MGIEasy DNA Adapters-16 (1000005284), MGIEasy Circularization Module (1000005260), MGISEQ-2000RS High-throughput Sequencing Kit (PE100), MGISEQ-2000RS Sequencing Chip (PE100), Qubit 1 × dsDNA HS Assay Kit (Q33231), Qubit RNA HS Assay Kit (Q32852).

2.3.2. Instruments

High-throughput tissue grinder (SCIENTZ-48), nucleic acid extraction instrument (GeneRotex 96), MGI high-throughput gene sequencer (MGISEQ-2000RS), MGI sample preparation system (MGISP-100), Qubit 4 Fluorometer (Waltham), real-time fluorescent quantitative PCR analyzer (QuantStudio™ 5), PCR amplification instrument (ProFlex™), and autoclave (MSL-3750).

2.4. Sample preprocessing and nucleic acid extraction

RNA was extracted from blood samples using the RNAsimple Total RNA Extraction Kit; tissue samples were homogenized and processed using RNAprep pure grinding, followed by centrifugation in a refrigerated centrifuge to obtain fecal supernatant. RNA was then extracted from the supernatant using the QIAamp Viral RNA Mini Kit; the experimental procedures were carried out according to the kit instructions. All samples underwent automatic nucleic acid extraction using the Tiangen pre-packaged nucleic acid extraction kit and nucleic acid extraction instrument.

2.5. Real-time fluorescent quantitative reverse transcription PCR (Real-time RT-PCR) and sequencing

Nucleic acids were tested for enterovirus-specific fluorescent quantitative polymerase chain reaction (Real-time PCR) using the One Step PrimeScript™ RT-PCR Kit. The experimental procedures were carried out according to the kit instructions, with the following reaction conditions: 42 ℃ for 10 minutes, one cycle; 95 ℃ for 1 minute, one cycle; 95 ℃ for 15 seconds, 50 ℃ for 30 seconds, and 72 ℃ for 1 minute, five cycles; 95 ℃ for 10 seconds, 55 ℃ for 40 seconds (collecting fluorescence signals), 40 cycles. After the reaction, read the CT value. Samples with a CT value greater than 35 were considered positive, and those with a CT value still less than 35 after re-evaluation were deemed negative. Positive samples underwent RT-PCR using the PrimeScript™ One Step RT-PCR Kit Ver.2, with universal enterovirus primers E486/E488 and E490/E492. The PCR products were purified and sequenced using the Sanger method, with sequencing performed by Beijing Boyunhui Biotechnology Co., Ltd.

2.6. High-throughput sequencing

2.6.1. Nucleic acid concentration measurement

To explore the composition of the pathogen spectrum in the specimens, libraries were constructed for each tissue specimen separately. The RNA concentration in the extracted samples was measured using the Qubit4 fluorometer. Specimens with a total nucleic acid content greater than 200 ng were selected for transcriptome sequencing (RNA Sequencing, RNA-seq). When the nucleic acid content in the specimens was below the detectable limit of the Qubit4 fluorometer, there was a risk of library construction failure.

2.6.2. Library construction

For specimens that meet the requirements, the MGIEasy-rRNA Removal Kit was used in conjunction with the MGI Sample Preparation System MGISP-100 to remove rRNA, thereby reducing the proportion of rRNA and increasing the amount of effective data in the raw sequencing output later on. The MGIEasy RNA Library Preparation Kit was used to fragment the nucleic acids, and the fragmented RNA underwent random reverse transcription to become cDNA. Subsequently, second-strand synthesis was performed, followed by end repair at both ends of the DNA fragments and ligation of sequencing adapters. The entire product was then subjected to magnetic bead selection to complete library construction.

2.6.3. DNB preparation, loading, and sequencing

The MGISEQ-2000RS High-Throughput Sequencing Kit was used to prepare the library into DNBs (DNA Nanoballs). The loading instrument was then employed to load the DNBs onto the MGISEQ-2000RS sequencing chip. After loading, the sequencing chip was left to stand still and wait for sequencing. Sequencing

was performed using the MGISEQ-2000RS from MGI.

2.7. Bioinformatics analysis

The results of high-throughput sequencing underwent quality control using Trimmomatic software, and the quality control results were inspected using FastQC. Bowtie2 was used to align the data with the human reference genome (hg19 was selected as the reference genome) to remove human genomic data. Simultaneously, the data were compared with viral reference databases, nucleotide databases, and non-redundant protein databases to determine the composition of the data. Finally, De novo assembly was performed using Trinity and Megahit, and the optimal assembly result was obtained through comparison with Quast. The assembled contig sequences were compared with the reference viral genome, and the resulting scaffolds were aligned with the NCBI NT database to obtain the assembled sequence information.

3. Results

3.1. Results of real-time fluorescent quantitative reverse transcription PCR and virus isolation

Among the 101 fecal samples tested by real-time fluorescent quantitative reverse transcription PCR (real-time PCR), 4 samples yielded positive results with CT values of 19.04, 24.56, 27.15, and 30.26, respectively. The PCR products were sent to Sangon Biotech (Shanghai) for sequencing, and no specific fragments of enteroviruses were detected. 5 fecal samples and 7 gastric contents from autopsy specimens were inoculated into RD cells, and no cytopathic effects (CPE) were observed after two blind passages.

3.2. Results of second-generation sequencing of autopsy samples

3.2.1. Quality inspection results of the fluorescence quantifier

After Qubit4 fluorescence quantification quality inspection of 50 autopsy samples, 10 samples were found to be below the lower limit of nucleic acid quantification detection concentration, posing a risk of library construction failure. Therefore, library construction was halted for these samples. Library construction was initiated for the remaining 40 samples that met the concentration requirements, among which library construction failed for 11 samples and succeeded for 29 samples. The 29 samples originated from 11 sudden death cases across 10 outbreaks, with the following sample types and quantities: 6 cardiac cavity blood samples, 5 liver samples, 3 fecal samples, 3 gastric content samples, 2 myocardial samples, 2 gastrocnemius muscle samples, 2 intercostal muscle samples, 2 kidney samples, 1 blood sample, 1 lung sample, 1 rib sample, and 1 spleen sample (**Table 1**).

Table 1. Information table of samples with successful database construction

Collection date	Collection site	Name	Gender	Age	Ethnicity	Sample type
July 2002	XMS Village	LXM	Female	18	Yi	Lung, Myocardium, Liver
July 2006	AJJ Village	LCX	Female	28	Yi	Liver
July 2007	XSD Village	WSC	Female	39	Han	Right Kidney, Myocardium, Left Liver, Right Liver, Left Kidney
August 2007	MCP Village	SJZ	Female	45	Han	Cardiac Chamber Blood
August 2007	HP Village	CDR	Male	31	Lisu	Feces

Table 1 (Continued)

Collection date	Collection site	Name	Gender	Age	Ethnicity	Sample type
July 2008	GXZ Village	YJF	Female	42	Yi	Cardiac Chamber Blood
August 2009	SGZ Village	ZLS-1	Female	29	Yi	Cardiac Chamber Blood
August 2009	WJC Village	WJP	Male	27	Yi	Cardiac Chamber Blood, Rib, Liver, Gastric Contents
July 2015	GC Village	LSF	Female	38	Han	Gastric Contents
July 2015	QSH Village	LM	Female	5	Han	Blood, Cardiac Chamber Blood, Feces, Gastrocnemius, Intercostal Muscle
July 2015	QSH Village	LXL	Female	28	Yi	Cardiac Chamber Blood, Blood, Gastric Contents, Gastrocnemius, Intercostal Muscle, Feces

3.2.2. Genome annotation results

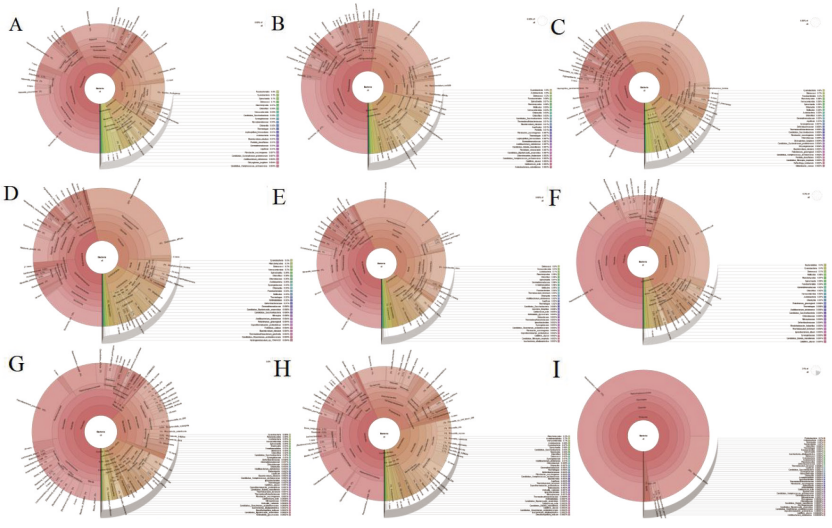
After high-throughput sequencing of 29 samples, a total of 50,589,250,206 valid Raw-reads were obtained, with sequencing data quality (Q30) ranging from 85.47% to 90.69%. The sequencing depth was sufficient for subsequent analysis. Reads were assembled into Contigs, and the resulting Scaffolds were compared with the NCBI Nucleotide database to obtain assembled sequence information. The annotation results revealed that for 10 samples, the predominant detected abundance was *Paeniclostridium sordellii* and its subspecies, while for 9 samples, *Escherichia coli* was the most abundant. Viral species were not successfully assembled due to their low abundance.

3.2.3. Species information and abundance of enterovirus read

Among the 29 samples with successful library construction, low-abundance enterovirus reads were detected in 11 samples (all with relative abundances $\leqslant$ 0.910%). Enterovirus A114 was detected in 6 samples, originating from the myocardium and left kidney of sudden death victim WSC (with relative abundances of 0.211% and 0.571%, respectively), the cardiac cavity blood and rib of sudden death victim WJP (with relative abundances of 0.910% and 0.013%, respectively), and the cardiac cavity blood and blood of sudden death victim LXL (with relative abundances of 0.002% and 0.0000263%, respectively). Coxsackievirus A2 was detected in 9 samples, originating from the myocardium, left kidney, and right kidney of sudden death victim WSC (with relative abundances of 0.111%, 0.192%, and 0.051%, respectively), the cardiac cavity blood and rib of sudden death victim WJP (with relative abundances of 0.291% and 0.007%, respectively), the gastrocnemius muscle and intercostal muscle of sudden death victim LM (with relative abundances of 0.00019% and 0.00342%, respectively), and the cardiac cavity blood and blood of sudden death victim LXL (with relative abundances of 0.000551% and 0.0000368%, respectively). Coxsackievirus B3 was detected in 9 samples, originating from the myocardium, left kidney, and right kidney of sudden death victim WSC (with relative abundances of 0.312%, 0.486%, and 0.120%, respectively), the cardiac cavity blood, rib, and liver of sudden death victim WJP (with relative abundances of 0.765%, 0.001%, and 0.001%, respectively), the cardiac cavity blood and blood of sudden death victim LXL (with relative abundances of 0.001% and 0.0000999%, respectively), and the gastric contents of sudden death victim LSF (with a relative abundance of 0.00000848%). The coexistence of 2 to 3 enteroviruses was observed in some samples (**Table 2**, **Figure 1**).

Table 2. Species and abundance of enterovirus read in 11 autopsy samples

Name	Sample type	Enterovirus species	Reads count	Relative abundance (%)
WSC	Myocardium	Enterovirus A114	87	0.312
		Coxsackievirus B3	59	0.211
		Coxsackievirus A2	31	0.111
	Left kidney	Enterovirus A114	182	0.574
		Coxsackievirus B3	154	0.486
		Coxsackievirus A2	61	0.192
	Right kidney	Coxsackievirus B3	73	0.120
		Coxsackievirus A2	31	0.051
	Cardiac chamber blood	Enterovirus A114	269	0.910
		Coxsackievirus B3	226	0.765
		Coxsackievirus A2	86	0.291
WJP	Rib	Enterovirus A114	10	0.013
		Coxsackievirus A2	5	0.007
		Coxsackievirus B3	1	0.001
LM	Liver	Coxsackievirus B3	1	0.001
	Gastrocnemius	Coxsackievirus A2	1	0.00019
	Intercostal muscle	Coxsackievirus A2	2	0.000342
LXL	Cardiac chamber blood	Enterovirus A114	186	0.002
		Coxsackievirus B3	164	0.001
		Coxsackievirus A2	65	0.000551
	Blood	Coxsackievirus B3	19	0.0000999
		Coxsackievirus A2	7	0.0000368
		Enterovirus A114	5	0.0000263
LSF	Gastric contents	Coxsackievirus B3	4	0.00000848



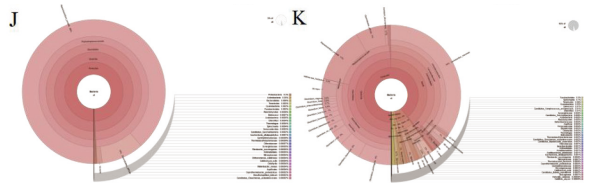


Figure 1. Composition of pathogenic species in 11 autopsy samples (A: WSC myocardium; B: WSC left kidney; C: WSC right kidney; D: WJP myocardium; E: WJP left kidney; F: WJP right kidney; G: LM gastrocnemius muscle; H: LM intercostal muscle; I: LXL cardiac chamber blood; J: LXL blood; K: LSF gastric contents).

4. Discussion

Yunnan sudden death has long been one of the severe endemic public health issues in our province, characterized by a high potential incidence of heart disease with diverse causes potentially linked to past viral infections, geological background, hygiene conditions, lifestyle habits, and genetic factors. In recent years, the implementation of comprehensive prevention and control measures has significantly reduced the incidence of Yunnan sudden death, yielding notable economic and social benefits. Virological studies were conducted on 33 Yunnan sudden death incidents occurring between 1978 and 2006, with 56 enterovirus strains isolated from 9 of these incidents (27 strains of CVA9, 10 strains of CVB3, 9 strains of CVB4, 7 strains of CVB6, 1 strain of CVA1, 1 strain of ECHO12, and 1 strain of a novel enterovirus) [2,3]. Tang Xue et al. conducted CVB antibody testing on serum samples from populations in Yunnan sudden death-affected areas, revealing a higher overall positive rate in the affected population compared to the control group [4]. In 2004, a “novel enterovirus” strain was isolated from a Yunnan sudden death-affected area, which was later identified as the enterovirus EV-B83 strain (GenBank accession number No. OQ990312) [5]. Previous research findings indicate that enterovirus infection may be one of the causes contributing to some cases of sudden death in Yunnan. Enterovirus A114, detected in this study, is a novel enterovirus with limited global research reports. Researchers believe that its recombination can lead to changes in virulence [6,7]. Coxsackievirus A2 is considered one of the common pathogens causing herpangina and hand, foot, and mouth disease (HFMD) in children. Infection can lead to central nervous system complications, cardiopulmonary failure, and viral myocarditis, with severe myocarditis being one of the primary causes of death resulting from CVA2 infection [8.9]. Coxsackievirus B3 is the main pathogen of viral myocarditis worldwide. As a highly cardiotropic virus, CVB3 infection can cause a series of severe cardiac diseases, including fulminant myocarditis, dilated cardiomyopathy, and even heart failure, and is also one of the factors contributing to some cases of sudden death in Yunnan [10-12]. The research results show that the autopsied organs of individuals who died suddenly in the affected areas carry fragments of various enteroviruses to varying degrees, suggesting that there may be varying degrees of enterovirus infection among the population in the Yunnan sudden death areas. Based on the detection results, this further validates the viral infection theory of sudden death in Yunnan and suggests a possible etiological association. *Paeniclostridium sordellii* and *Escherichia coli* are widely present in soil, sewage, animal, and human intestines. The detection of these two bacteria in this study may be attributed to contamination from normal intestinal flora or the autopsy sampling process.

The application of metagenomics next-generation sequencing (mNGS) has revolutionized traditional clinical diagnostics, garnering significant attention and widespread use in fields such as central nervous system infections, bloodstream infections, respiratory infections, gastrointestinal infections, and ocular

infections [13–16]. Currently, there are relatively few research reports on backward reasoning of causes of death through postmortem pathogen detection. However, based on a review of relevant studies on COVID-19 infectious corpses, this method, despite its operational challenges, holds substantial practical value for forensic pathology, cause-of-death identification, and understanding the spatiotemporal patterns of pathogen invasion in the human body [17]. Using autopsy specimens from cases of unexplained death, mNGS, as a retrospective tool, can facilitate the identification or exclusion of suspected pathogens in public health emergencies in future work. This study integrated metagenomics next-generation sequencing, real-time fluorescent quantitative reverse transcription PCR, and traditional virological isolation methods to conduct a comprehensive, multidimensional exploration of samples from populations in Yunnan sudden death areas. This approach represents an innovation in the history of Yunnan sudden death research and offers practicality and potential for broader application in etiological investigations.

The limitations of this study include the extreme difficulty in conducting autopsies, resulting in a small sample size and thus only allowing for descriptive research analysis. Additionally, the low abundance of enterovirus reads in the samples prevented successful assembly, hindering in-depth analysis of related pathogens. Moreover, the prolonged storage of samples may have led to the degradation of pathogen genetic material or sampling contamination, potentially introducing bias into the detection data. However, based on a comprehensive review of previous studies, enteroviruses, as an objective biological factor, may play a complex role in the Yunnan sudden death incidents, ranging from background collaboration to direct causation. Nevertheless, their specific pathogenic mechanisms, particularly the synergistic or cascading effects under the coexistence of multiple viruses, remain to be elucidated through subsequent research.

5. Conclusion

In summary, enterovirus infection is potentially a contributing factor to certain unexplained sudden deaths in Yunnan. It is also plausible that varying degrees of enterovirus infection may be present within some populations of the affected regions. Meanwhile, the detection of bacteria in these cases is likely attributable either to the normal human intestinal flora or to contamination during the autopsy sampling procedure.

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Disclosure statement

The authors declare no conflict of interest.

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