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RESEARCH ARTICLE

Molecular Detection of Virulence and Drug Resistance Genes of Pathogenic *Escherichia coli* from Calves in Chongqing, China

Zhonghua Liao[1,2], Xueyan Chen[1,2], Zhenjing Li[1,2], Yuan Gao[1,2] and Shijun Hu[1,2,*]

[1]School of Animal Science, Rongchang Campus, Southwest University, 160 Xueyuan Road, Rongchang District, Chongqing, 402460, China; [2]Veterinary Science Engineering Research Center of Chongqing, 160 Xueyuan Road, Rong chang District, Chongqing, 402460, China
*Corresponding author: 435277784@qq.com

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A B S T R A C T

The aim of this study was to detect virulence genes and drug resistance genes in pathogenic *Escherichia coli* (*E.coli*) isolated from diarrhea calves feces and to detect drug resistance phenotypes, and genotypic. As a result, a total of 22 strains of *E.coli* were separated and identified, but only 18 strains were pathogenic. The virulence gene test showed that the LT, Stx1, F5, F6, and irp2 gene detection rate were 66.67% (12/18), 16.67% (3/18), 55.56% (10/18), 44.44% (8/18) and 16.67% (3/18), respectively. Antibiotic susceptibility tests showed that the isolates were susceptible to Kanamycin and Florfenicol and resistant to Cefalotin (94.44%) and the like. Resistance gene tet (A), gryA, parC, sulI, sulII, sulIII, blaTEM, blaCTX-m, blaSHV, aac (3)-II, aac (6 ')-Ib and rmtB detection rate are 77.78% (14/18), 44.44% (8/18), 55.56% (10/18), 22.22% (4/18), 11.11% (2/18), 11.11% (2/18), 77.78 % (14/18), 5.56% (1/18), 44.44% (8/18), 50% (9/18), 55.56% (10/18), and 27.78% (5/18), respectively. ERIC-PCR (repetitive intergenic consensus-polymerase chain reaction) classified 18 pathogenic *E.coli* into 4 distinct genotypes, and all strains carrying the LT virulence gene were brought together, but there was no link between drug resistance genes and genotypic. The results of this study provids experimental evidence for the prevention, control pathogenic *E. coli* disease in calves.

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INTRODUCTION

E.coli is the most common pathogen of bacterial diseases in the livestock industry. Depending on research reports, in cattle-borne diseases, pathogenic *E. coli* with high infection rate is enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E.coli* (EHEC) (Miraglia *et al*., 2001). ETEC is the most frequent pathogen of calf diarrhea, which is more severe in Asia (Ori *et al*., 2018). It has two main virulence factors: F5、F41 and heat-resistant and heat-labile enterotoxin. EHEC is a variant of Shiga toxin-producing *E.coli* (STEC) that produces Shigella toxin. STEC is associated with massive diarrhea, hemorrhagic enteritis, and hemolytic uremia (Ori *et al*., 2018). There are many serotypes in *E. coli*. It has been reported that uropathogenic *E. coli* is associated with O1、O2、O6、O16、O18、O25、O75 but serotypes cannot be used to determine the pathogenicity of *E. coli* (Kim *et al*., 2018). The pathogenic of *E. coli* mainly depends on the specific virulence factors it produces, such as fimbrae, iron uptake systems and toxins.

In order to prevent disease and promote growth, β-lactams, tetracyclines and other antibiotics are often used in the cattle industry, thereby increasing the production of drug-resistant bacteria. It was reported that resistant bacteria could be passed from animals to humans through contact (Begum *et al*., 2018). *E. coli* is a common intestinal microflora in human and animal. Once it develops resistance, it will cause serious harm.

ERIC sequence was discovered by Hulton *et al*. (2010) in *E. coli*, Salmonella typhimurium and other gut bacterial genomes, the sequence was 126bp long and its position on the chromosome and copy number was species specific. ERIC-PCR technology was commonly used in strain identification and traceability research of pathogens because of its accuracy, simplicity, rapidity, and economy (Prabhu *et al*., 2010).

In the current study, was intended to understand the virulence genes and drug resistance of pathogenic *E. coli* in calves, and the relationship with molecular typing. This study collected fecal samples from diarrhea calves in Chongqing, China, isolated and identified the pathogenic *E.coli*, and tested virulence genes, drug resistance genes, drug susceptibility and genotyping. Provide a reference to the prevention and treatment of various diseases caused by pathogenic *E. coli*.

MATERIALS AND METHODS

Ethics statement: The collection of feces samples had been approved by the owner of the farm. Conduct animal experiments in accordance with laboratory regulations. This study was approved by the Ethics Committee of Southwest University.

Isolation and identification of bacteria: A total of 30 calf diarrhea fecal samples were aseptically collected from different cattle farms in Fengdu County, Chongqing. The samples were inoculated in 1ml LB liquid medium (Beijing Aoboxing Co., Ltd, Beijing, China) for 24 hours at 37°C. Then they were inoculated separately on MacConkey agar medium (Beijing Aoboxing Co., Ltd, Beijing, China). Select a single colony of pink on MacConkey agar medium. Final determination of whether it is *E. coli*, mainly by PCR to determine whether the strain carries the *E. coli*-specific PhoA gene.

Pathogenicity test: All isolates were verified for pathogenicity by mice pathogenicity test. Each strain was inoculated intraperitoneally in three mice, a control group injected with saline. Each mouse weighed 20-22g (Kunming mice, KM), each inoculated with 0.2 ml (0.5×10^9 CFU/mL). Observe for 24 hours and note the results, necropsy of dead mice.

Virulence gene detection: Direct extraction of purified *E. coli* DNA with Lysis Buffer for Microorganism to Direct PCR (TAKALA, Beijing, China), stored at -20°C. Pathogenic isolates were used to detect major virulence genes, including STa, STb, LT, Stx1, Stx2, Stx2e, eaeA, Irp2, F4 (K88), F5 (K99), F6 (987P), F41, F18 gene. All PCR reaction systems and reaction conditions were in accordance with the literature protocol, STa, STb, LT, F4 (K88), F5 (K99), F6 (987P) gene (Bosworth and Casey., 1997); Stx1, Stx2, eaeA gene (Sidhu *et al*., 2013); Irp2, Stx2e, F41, F18 gene (Zhang *et al*., 2007). All primers were synthesized by Shanghai Biotech Co., Ltd (Shanghai, China).

Antimicrobial susceptibility test: The susceptibility test was carried out on the isolates by the disk diffusion method, and the test method accord to the Clinical and Laboratory Standards Institute's guidelines（CLSI）. We selected a total of 10 kinds of antibiotics for susceptibility testing, including Florfenicol (FLR, 30 ug/disk), Tobramycin (TOB, 10 ug /disk), Cefalotin (CEP, 30 ug/disk), Mezlocillin (MEZ, 75 ug/disk), Doxycycline (DOX, 30 ug/disk), Norfloxacin (NOR, 10 ug/disk), Enrofloxacin (ENR, 5 ug/disk), kanamycin (KAN, 30 u/disk), Amoxicillin (AMX, 10 ug/disk), Trimethoprim/ Sulfamethoxazole (SXT, 1.25/23.75 ug/disk). All Drug sensitive paper tablets were purchased from Hangzhou Binhe Microbiology Co., Ltd (Hangzhou, China). The drug susceptibility test results were analyzed by WHONET 5.6 software.

Antibiotic resistance gene detection: A total of 17 drug resistance genes were tested by PCR. Tetracycline resistance gene: tet(A), tet(B); quinolones resistance gene: gyrA, parC; sulfonamides resistance gene: sulI, sulII, sulIII ; beta-lactams resistance gene: blaTEM, blaCTX-m, blaSHV; aminoglycosides resistance gene: Arm, RmtB, aac(6')-Ib, aac(3)-II, ant(3")-I; amphenicols resistance gene: floR, ClmA. The PCR reaction conditions and reaction system were carried out according to the corresponding reference (Yu *et al*., 2017).

ERIC-PCR genotyping of pathogenic isolates: Molecular typing of pathogenic *E. coli* by using ERIC-PCR, amplification primer was ERIC:5'- AAGTAAGT GACTGGGGTGAGCG-3', the PCR reaction conditions and reaction system were carried out according to the corresponding reference (Li *et al*., 2011). The amplified product was electrophoresis on a 1.3% agarose gel. Electrophoresis at 100 V for 30 min and the image was processed with a gel imaging system (BIO-RAD, US). Clustering analysis of ERIC-PCR results used Gel-Pro Analyzer 4.0 and NTSYS pc 2.1 software (Yamamoto *et al*., 2014).

RESULTS

***E.coli* isolation and pathogenicity results:** A total of 22 strains of *E. coli* were identified by PCR amplification. Mouse pathogenicity test results showed: no change in the control mice, the mice in the experimental group developed symptoms such as lethargy and anorexia after injection of *E. coli*. A total of 18 strains of *E. coli* caused death in mice, anatomical dead mice showed yellow exudate in the abdominal cavity; pulmonary congestion, hemorrhage; liver congestion, bleeding; splenomegaly, necrosis; gassing of the stomach and intestines.

Virulence gene test results: The results showed five virulence genes were detected, namely LT, Stx1, F5, F6, irp2. The detection rates of LT, Stx1, F5, F6, and irp2 genes were 66.67% (12/18), 16.67% (3/18), 55.56% (10/18), 44.44% (8/18) and 16.67% (3/18), respectively. There were three strains carrying LT, F5 gene, two strains carrying LT, F6 gene, three strains carrying LT, F5, F6 gene, and three strains carrying LT, irp2, F5, F6 gene. These strains containing the LT considered to be ETEC, containing the Stx-1 gene were classified as EHEC. As a result, twelve pathogenic *E. coli* were classified as ETEC, three pathogenic *E. coli* were classified as EHEC and three strains were not considered (Table 1).

Antibiotic susceptibility test results: The experimental results were analyzed by the WHONET5.6 software and the results showed resistance was most frequently detected against CEP (94.44%) followed by MEZ (66.67%), DOX (11.11%), AMX (44.44%), NOR

(38.89%), SXT (27.78%), TOB (16.67%), ENR (5.56%), KAN and FLR were sensitive. All strains showed multi-drug resistance, producing 14 kinds of antimicrobial spectrum. Resistant to 2, 3, 4, 5 and 6 antibiotics had 1, 5, 6, 2 and 4 strains, respectively (Table 2).

Drug resistance gene test results: Only tet (A) was detected in two tetracycline resistance genes, the detection rate was 77.78% (14/18). Among the quinolone resistance genes detected, the detection rate of the parC gene was higher at 55.56% (10/18). The detection rate of the gryA gene was lower at 44.44% (8/18). The three sulfonamide-resistant genes tested, the SulI gene was the most prevalent, 22.22% (4/18), followed by the SulII, SulIII gene, and the detection rate was the same, which was 11.11% (2/18). The detection rates of blaTEM, blaCTX-m, and blaSHV were 77.78% (14/18), 5.56% (1/18), 44.44% (8/18), respectively. The detection rates of aac(3)-II, rmtB, and aac(6')-Ib were 50%(9/18), 27.78%(5/18), and 55.56%(10/18), respectively. The amphenicols resistance gene was not detected. All strains carried drug resistance genes, up to eight drug resistance genes.

Table 1: Type of pathogenic *E. coli*

Combination of genes	Pathotypes	Total (%)
LT	ETEC	1(5.56)
LT, F5		3(16.67)
LT, F6		2(11.11)
LT, F5, F6		3(16.67)
LT, irp2, F5, F6		3(16.67)
Stx1	EHEC	2(11.11)
Stx1, F5		1(5.56)
Non-detected		3(16.67)
Total		18

Table 2: Pathogenic *E. coli* antimicrobial spectrum

Type of resistance	Antimicrobial spectrum	Number of strains
2	MEZ+CEP	1
3	MEZ+CEP+DOX	2
	SXT+MEZ+CEP	1
	AMX+MEZ+CEP	2
4	MEZ+CEP+ENR+DOX	1
	MEZ+CEP+KAN+NOR	1
	SXT+MEZ+CEP+DOX	2
	AMX+MEZ+CEP+NOR	2
5	AMX+MEZ+CEP+KAN+NOR	1
	AMX+SXT+MEZ+CEP+DOX	1
6	AMX+MEZ+CEP+KAN+NOR+ENR	1
	AMX+MEZ+CEP+TOB+NOR+ENR	1
	AMX+MEZ+CEP+TOB+NOR+ENR	1
	AMX+SXT+MEZ+CEP+TOB+NOR	1

Comparative analysis of drug resistance genes and drug resistance phenotypes, tet(A) genes were detected in 14 strains of 18 strains pathogenic *E. coli*, and 2 of them were resistant to DOX, the coincidence rate of drug resistance gene and drug resistance phenotype was 14.29% (2/14). The coincidence rate of the amphenicols resistance gene and the resistant phenotype was 100%. The coincidence rates of ENR and TOB resistant phenotypes and drug resistance genes were 10% (1/10), 12.50% (3/24). Detailed results were given in Table 3.

ERIC-PCR molecular typing results: Per isolates produced 1-11 fragments, ranging in size from 100bp-2000bp. As shown in Fig. 1, 18 strains of pathogenic *E. coli* was divided into 4 kinds of genotypes (I-IV), genetic similarity was between 55% and 100%. TypeI had 7 strains pathogenic *E. coli*, including fd1, fd3, fd5, fd15, fd12, fd13, fd14; typeII had 5 strains pathogenic *E. coli*, including fd2, fd4, fd8, fd11, fd9; typeIII had 2 strains pathogenic *E. coli*, including fd16, fd17; typeIV had 4 strains pathogenic *E. coli*, including fd10, fd6, fd7, fd18 (Fig. 1). I, II and III genotypes were clustered in the upper half of the tree. Except for fd5 and fd17, all contained the virulence gene LT. Moreover, genotype IV was in the lower half of the tree, except that fd10 did not contain the stx1 gene, and fd6, fd7, fd18 contained the stx1 gene. However, strains with the same genotype had different resistance genes.

DISCUSSION

The prevalence of calves with diarrhea in China has been expanding and has been reported in Henan, Shanxi, Heilongjiang and other regions. In intensively managed farms, the incidence and severity of calves with diarrhea are increasing, which may be the result of a large number of infections caused by pathogenic *E. coli*. To distinguish whether it is a pathogenic strain, this study was associated with animal experiments to identify it. Ghanbarpour *et al*. (2017) reported that the prevalence of pathogenic *E. coli* in Iranian lambs reached 71.66%. Andrade *et al.* (2012) reported that pathogenic *E. coli* detected in 59.26% of calves fecal samples in Minas Gerais, Brazil. In this study, 22 strains of *E. coli* were isolated from 30 feces of calves with diarrhea, and 18 strains were pathogenic, with a detection rate of 60%. So, the detection rate of pathogenic *E.coli* varies differs from region to region.

Table 3: Comparison of coincidence rates between pathogenic *E. coli* resistance genes and drug resistant phenotypes

Antibiotic type	Drug resistance gene	Number of positive strains	Antibacterial drugs	Number of positive phenotypes	Compliance rate %
Tetracycline	tet(A)	14	DOX	2	14.29
Quinolone	parC	10	ENR	1	10.00
			NOR	7	70.00
	gryA	8	ENR	1	12.50
			NOR	7	87.50
Sulfonamide	sul Ⅰ or sul Ⅱ or sul Ⅲ	8	SXT	5	62.50
β-lactam	blaTEM or blaCTX-M or blaSHV	23	AMX	8	34.78
			MEZ	12	52.17
			CEP	17	73.91
Aminoglycoside	aac(3)-Ⅱ or rmtB or aac(6')-Ib	24	TOB	3	12.50
Amphenicols	floR, ClmA	0	FLR	0	100.00

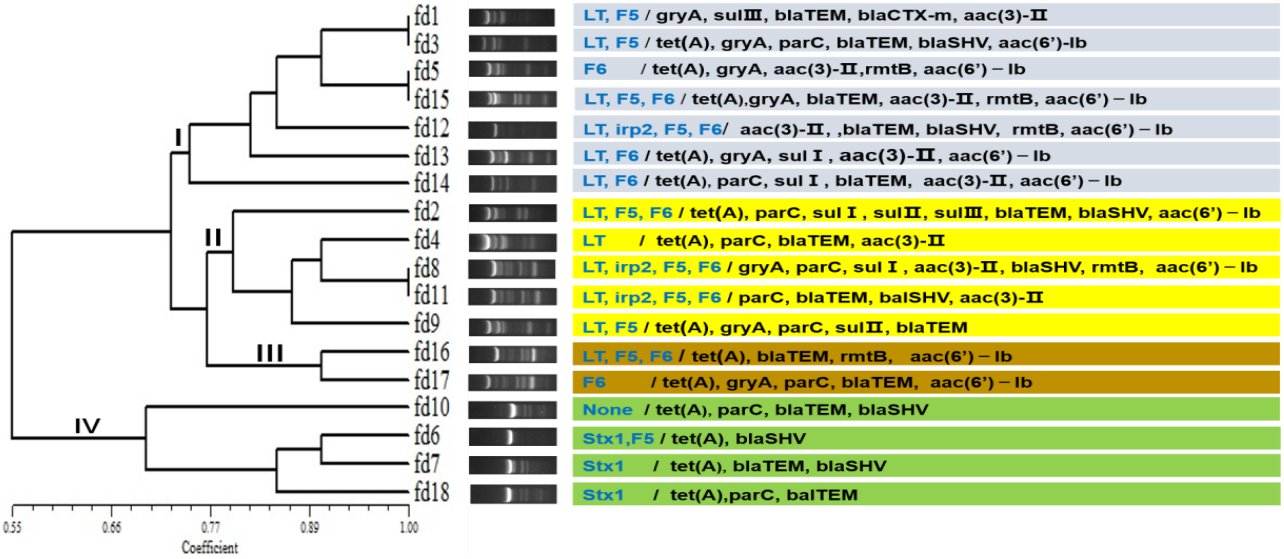

Fig. 1: ERIC-PCR typing results of 18 strains of calves pathogenic *E. coli*.

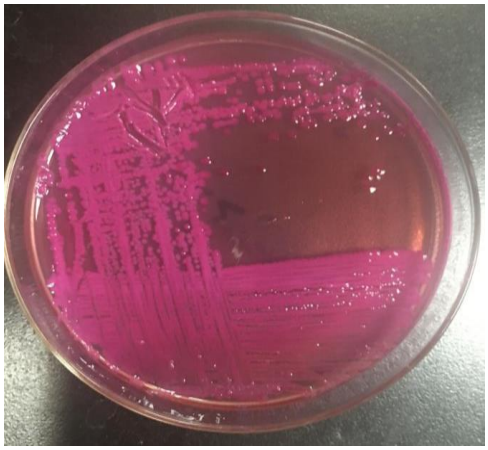

Fig. 2: The morphology of *E.coli* on MacConkey agar medium.

It can be observed in the test results of 18 strains of pathogenic calves *E.coli* virulence genes that all strains except fd10 contain one or several virulence genes. The detection rates of virulence genes LT, Stx1, F5, F6, and irp2 were 66.67%, 16.67%, 55.56%, 44.44% and 16.67%, respectively. Andrade *et al*. (2012) reported Stx1 and F41were detected in diarrhea calves feces, with detection rates of 70% and 30%. Hakim *et al*. (2017) reported that 14 strains of *E.coli* virulence genes were detected, resulting the detection rates of Stx1 and Stx2 genes were 42.86% and 28.57%, respectively. LT and F5 genes are markers of ETEC, indicating that the pathogenic *E.coli* isolated from this study was mainly ETEC, LT, F5, and F6 are dominant virulence genes. However, no F6 gene had been declared in cattle source EHEC. The pathogenic strain of newborn calves has been shown to be EHEC and is often isolated from diarrhea calves (Yongil *et al*., 2014). Bako *et al*. (2017) investigated diarrhea *E.coli* on feces, slurry, etc. in the cattle market, and ETEC was the most popular. At present, there are many reports on the detection of LT genes by human and pig source ETEC, and there are few reports on the detection of LT genes by bovine ETEC, however, all of the bovine ETEC isolated in this experiment carries the LT gene. High pathogenicity island (HPI) is an important virulence factor of *E. coli*, in which irp2 is a marker gene of HPI. Three strains of pathogenic *E. coli* contain the irp2 gene in this study, indicating that HPI is also prevalent in bovine *E. coli*.

The susceptibility results of this test, 18 strains pathogenic calves *E. coli* show different levels of resistance to most antibiotics.Some strains were resistant to six antibiotics, and most strains were resistant to three and four antibiotics. Resistance to cefotaxime exceeds 94%, relatively sensitive to enrofloxacin and florfenicol. Shahrani *et al*. (2014) reported that the resistance rates of cefotaxime, and enrofloxacin in *E. coli* isolated from diarrhea calf feces were 52.06%, and 61.42%, respectively. Hakim *et al*. (2017) reported that the resistance rate of isolated *E. coli* , ampicillin was 71.4%, sulphaprim was 50%, it was highly sensitive to norfloxacin, trimethoprim-sulfamethoxazole, and cephalosporin. These results may indicate a difference in drug resistance between different pathogenic *E. coli*, and susceptibility testing should be performed first when antibiotics are used to treat *E. coli* type diseases. In the study, isolated strains have 14 antimicrobial spectrum. Yamamoto *et al*. (2014) analyzed the resistance of *E. coli* from four farms in Japan, resulting in 8 drug resistance profiles. These results indicate that there are a lot of antimicrobial spectrum and multi-drug resistance in bovine *E. coli*.

Most of the resistance genes were highly prevalent in the tested strains, indicating that these genes play a major role in coding the resistance of the tested strains. In this study, tetracycline-resistant genes are mainly encoded by tet (A), which is consistent with the results of Fan *et al*. (2014). The experimental results suggest that active efflux is still the main mechanism of *E. coli* resistance to tetracyclines. The production of quinolone resistance is mainly related to the DNA helicase and topoisomerase IV of amino acid mutation in cells, and the mutations of gyrA and parC play a major role in drug resistance, consistent with the results of this study. Sulfonamide-resistant genes are mainly encoded by sulI, sulII, and sulIII, the sulII detection rate in this study is greater than that of sulI and sulIII, which is consistent with the results of Mohsin *et al*. (2017). Plasmid-mediated extended-spectrum β-lactamases (ESBLs) are the main cause of resistance to a new

generation of β-lactamase antibiotics. The results show that the blaTEM gene has the highest detection rate, which is consistent with the results of Tang *et al*. (2011). In this study, aminoglycoside resistance is mainly controlled by the aminoglycoside-modifying enzymes aac (3) -II, aac (6') -Ib and the methylase rmtB gene encoding 16S rRNA, and the former is dominant. Studies have reported that aac(6)-Ib is the most common G^- strain (accounting for more than 70% of all aac(6)-I gene detection rates) and is the most common acetylase gene in G^-. Resistance in G^- in European countries is often associated with aac(3)-II, aac(6)-I (Neonakis *et al*., 2003). Methylase detected in other Asian countries such as Korea were also dominated by rmtB (Gurung *et al*., 2010). In this study, the number of detections of each type of antibiotic resistance gene is higher than the number of resistant phenotypes. The cause may be linked to the expression level of drug resistance genes, the amount of enzyme produced by bacteria and the antibacterial activity of different antibacterial drugs and their differences in enzyme stability.

ERIC-PCR is a simple and rapid genotyping technique commonly used at present. Its resolution is sharp, and the results can be related to the "gold standard" PFGE classification of bacterial molecular biology typing technology. Versalovic *et al*. (1991) reported that finger-printing techniques could rapidly identify bacterial species and help analyze prokaryotic genomes. In this study, 18 strains of pathogenic *E. coli* is divided into 4 kinds of genotypes (I-IV). Sekhar *et al*. (2017) used ERIC-PCR to classify isolated virulent *E. coli*, and virulent *E.coli* was divided into three major clusters. Silveira *et al*. (2002) had pointed out that most pathogenic and non-pathogenic isolates were separated into different groups, indicating that each disease was caused by a specific genotype. Therefore, we combine the ERIC typing results with the virulence gene consequences, result all the LT genes are clustered and all the Stx-1 genes are clustered together. This may be because the same type of *E. coli* has a similar ERIC profile. Bessa *et al*. (2007) showed no direct correlation between genotype and resistant phenotype. Therefore, genotypic and drug resistance phenotypes are not analyzed, but a joint analysis of genotypic and drug resistance genes. The test results show that there is no link between drug resistance genes and genotypic.

Authors contribution: Zhonghua Liao and Zhenjing Li were responsible for the experiment, Xueyan Chen was responsible for writing the article, YG was responsible for processing the data, SH was the instructor.

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