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ARTICLE

Yersinia enterocolitica infection in diarrheal patients

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Abstract In this study, we hoped to provide valuable clinical information on yersiniosis for clinicians. Two thousand six hundred stool samples were collected from in- and outpatients with diarrhea, which were tested with both culture method and real-time polymerase chain reaction (RT-PCR). In total, 188 positive samples were detected by RT-PCR (178) and culture method (160), while the incidence was about 7.23%. The detection rate of RT-PCR was significantly higher than culture method and a higher incidence in autumn-winter was also noticeably identified than in spring-summer. Infection sources mostly focused on unboiled foods (101) and pets (45), while clinical manifestation mainly presented as gastroenteritis (156), pseudoappendicitis (32), and extraintestinal complications (46). The morbidity of extraintestinal complications in adults was significantly higher than in children and it was the same for high-risk patients between adults over the age of 60 years (4.7%) and children under the age of 3 years (1.4%), whereas the constituent ratio of children versus adults with yersiniosis in different systems was not significant. Of 160 isolates tested for antimicrobial susceptibility, the majority were susceptible to third-generation cephalosporins, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole, whereas only a small portion was susceptible to the first-generation cephalosporins and penicillins. During autumn-winter months, clinicians should pay more attention to clinical manifestation, early diagnosis, and treatment with susceptible antibiotics of yersiniosis and its complications, targeting high-risk patients.

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Introduction

Yersinia enterocolitica is a food-borne pathogen causing symptoms such as fever, diarrhea, nausea, vomit, and abdominal pain [1, 2], the diseases of which range from self-limiting gastroenteritis to fatal septicemia [2]. Extra-intestinal complications caused by virulent *Y. enterocolitica*, such as liver and spleen abscesses [3], pneumonia [4], septic arthritis [5], meningitis [6], empyema [7], and endocarditis [8], were widely reported. Human yersiniosis is attributed to contaminated pork, milk, water, and tofu consumption, as well as blood transfusion [2]. Patients may carry *Y. enterocolitica* in stools for 90 days after the resolution of symptoms [9], which suggests that the early detection of *Y. enterocolitica* from the diarrhea stool samples is critical in preventing its transmission and an eventual outbreak. However, clinicians' knowledge of the clinical presentation of yersiniosis and vigilance for this relatively rare disease may be insufficient, so yersiniosis is often neglected and cured ambiguously with insusceptible antibiotics, which increases the chances of extraintestinal complications and probably even leads to bad prognosis.

Sometimes, culture methods for the detection of *Y. enterocolitica* are time-consuming, unreliable, and unavailable in routine laboratories. Traditional polymerase chain reaction (PCR) assay is an effective way of detecting and identifying the organisms in animals and foods, including pathogenic *Y. enterocolitica* [10–14]. Multiplex PCR was used to confirm the presumptive *Y. enterocolitica* isolates cultured from pigs [15]. However, the common PCR method needs lengthy enrichment, amplification, and gel-based detection. Real-time PCR (RT-PCR) protocols no longer require gel-based detection, but they rely on the cleavage of a fluorescent probe for the automated and specific detection of amplicons, which allows for product

quantification. Recently, RT-PCR has been widely used to investigate the prevalence of *Y. enterocolitica* in swine feces, targeting the chromosomal attachment invasion locus-*ail* gene [16, 17].

In our former study [18], we found that RT-PCR was more efficient and time-saving than culture methods. RT-PCR was not only specific to virulent *Y. enterocolitica* with the very primer and probe [13], but it is also sensitive with the lowest detection limit in pure cultures and stool samples at about 100 CFU/ml and 1,000 CFU/g, respectively. Furthermore, the excellent consistency between RT-PCR and culture method (*Yersinia*-selective enrichment, CIN plate; YSE-CIN) was identified preliminarily. Based on the data above, we decided to choose RT-PCR as the investigational method and authenticate its consistency with culture method (YSE-CIN) using more samples.

In China, or even possibly some developed countries, physicians have paid little or no attention to yersiniosis and its complications since systemic clinical data may not exist for yersiniosis in the area. In our previous study [19], we carried out a preliminary and clinical investigation of *Y. enterocolitica* using 700 diarrhea stool samples, which only provided limited or possibly unreliable information on yersiniosis. However, in this study, 2,600 samples (188 positive) are relatively sufficient to draw a solid conclusion that may be helpful to doctors. Moreover, antimicrobial susceptibility tests and rare extraintestinal complications, as well as some statistic analysis that could not be completed due to the lack of positive samples previously, will be fully presented and discussed in this paper.

In this study, we hoped to carry out an epidemiological investigation of *Y. enterocolitica* from a clinical perspective, including data of infection sources (etiology), manifestation, complication, treatment, and prognosis, in order to provide valuable clinical information on yersiniosis for clinicians. Moreover, the reliability of RT-PCR, a relatively new method for the detection of *Y. enterocolitica* from stool samples, was also worthy of our investigation.

Materials and methods

Samples

Two thousand six hundred diarrhea stool samples (from 2,600 patients, males:females=1,540:1,060, children: adults=1,446:1,154, spring:summer:autumn:winter= 469:500:775:856) were randomly and continuously collected from May 2005 to January 2008 from in- and outpatients in Nanfang Hospital (1,600 samples), Zhujiang Hospital (500 samples), and Guangdong Provincial People's Hospital (500 samples), who defecated >3 times per day and accompanied by changes of stool character, e.g., being watery. All samples were used for clinical investigation with permission. The patient age ranged from 3 months to 83 years. Children were defined as <18 years and adults >18 years of age. All samples (about 1 g each) were kept in Cary-Blair solution (5 ml) and immediately sent to the laboratory. Yersiniosis is the infection of pathogenic *Y. enterocolitica* that can be detected by either RT-PCR or culture method besides clinical manifestations, the bio/serotype of which may be the following pathogenic serotypes: O:1,2a,3; O:2a,3; O:3; O:8; O:9; O:4,32; O:5,27; O:12,25; O:13a; O:13b; O:19; O:20, and O:21 [9].

Yersinia-selective enrichment, CIN plate and culture method

Yersinia-selective enrichment, CIN plate (YSE-CIN) and culture method were prepared and performed as previously described [18, 19].

Stool and blood examination, as well as antimicrobial susceptibility test

All stool samples were sent to the department of laboratory medicine for routine examination, while blood culture was also performed in the same department when patients presented as septicemia. All 160 isolates from the culture method were performed with antimicrobial susceptibility test in the department of laboratory medicine to choose the correct antibiotics treatment, recommended by doctors, following the National Committee for Clinical Laboratory Standards (NCCLS, 2005 edition) guidelines.

Bacterial strains and diagnostic serum

Serotypes O:3 and O:9 of *Y. enterocolitica* were used as reference strains. Diagnostic serum was purchased from Statens Serum Institut as previously [18, 19]. The serodiagnostic test was performed according to the manufacturer's instructions.

Classification of biogroup in *Y. enterocolitica*

The classification of six biogroups (1A, 1B, 2, 3, 4, and 5) was performed with biochemical tests (supplied by the Mérieux Foundation) according to the standard created by Wauters [1, 20].

Specific primer and probe, DNA extraction, and RT-PCR

The primer and probe sequences targeting the *yst* gene reported by Ibrahim et al. [13] were modified to suit the requirements of the assay and synthesized as in previous studies [18, 19]. Meanwhile, these primer and probe

sequences were tested and shown to be efficient in stool samples, besides in pure cultures [18, 19].

All stools were selectively enriched in YSE at 16°C for 18 h. A quantity of 200 μl of compound was pipetted into the microtube and DNA extraction was performed as previously [18, 19]. Finally, a 5-μl aliquot of extraction was served as the template for each RT-PCR amplification.

The RT-PCR components and conditions were the same as before [18, 19]. Each set of reactions included triplicate wells that were no-template controls, negative controls, and positive controls, respectively. The reaction was performed on an MJ Research Opticon 2 DNA Engine using the equation $\Delta RQ = RQ^{+} - RQ^{-}$ [10]. A positive interpretation for pathogenic *Y. enterocolitica* was based on a threshold of four times the average ΔRQ value of negative controls (stool without virulent *Y. enterocolitica*) [21], which enabled us to eliminate a lot of other bacteria that fell in the positive interpretation range.

Information collection

Basic information from outpatients, including name, sex, age, address, and so on, were recorded when they visited the doctors. Further information about their condition after treatment was also collected by telephone or e-mail. On the other hand, information from inpatients was acquired from their case histories and/or the attending physician with permission.

Statistical analysis

All statistical analysis (McNemer test, Chi-square test, and the Mann-Whitney *U*-test) were performed using the SPSS v13.0 software package by the Department of Statistics, Southern Medical University, Guangzhou, China.

Results

Positive samples and incidence analysis

All 2,600 diarrhea stools were tested by RT-PCR and culture method (YSE-CIN). The total number of positive samples was 188 (children:adults=128:60, males:females=106:82, citizens:famers=84:104, spring:summer:autumn:winter=25:20:58:85, Nanfang Hospital:Zhujiang Hospital:Guangdong Provincial People's Hospital=126:27:35). One hundred and seventy-eight positive samples were confirmed by RT-PCR, while 160 were identified by culture method.

The total incidence rate was about 7.23%, which was statistically higher than before (0.65%, 256/39,412, u=29.9, P<0.05) by the Mann-Whitney *U*-test.

The morbidity was not significant between males and females (u=0.86, P>0.05, Table 1) by the Mann-Whitney *U*-test, whereas it was significant between children and adults (u=3.52, P<0.05, Table 1), as well as between farmers and citizens (u=3.31, P<0.05, Table 1).

Significance was noted in the morbidity between autumn-winter and spring-summer months by the Mann-Whitney *U*-test (u=3.97, P<0.001, Table 2), while no significance was noted between the autumn and winter months (u=1.7, P>0.05). Additionally, the constituent ratio of children and adults in the four seasons was not significant by the Chi-square test (χ^2=5.07, P>0.05).

The incidence of yersiniosis in children under 3 years of age was significantly higher than in children aged from 3 to 18 years by the Mann-Whitney *U*-test (u=3.26, P<0.05, Table 3) and the incidence in adults over 60 years of age was also statistically higher than in adults aged from 18 to 60 years (u=3.0, P<0.05). Moreover, children under 3 and adults over 60 years of age are defined as a high-risk group because they are more susceptible to *Y. enterocolitica* infection. In the high risk group, the incidence in children under 3 years of age was significantly higher than in adults over 60 years of age by the Mann-Whitney *U*-test (u=2.6, P<0.05).

The morbidity of yersiniosis among the three hospitals was not significant by the Chi-square test (χ^2=5.2, P>0.05, Table 4), while the detection rate of RT-PCR was significantly higher than culture method by the McNemer test (χ^2=7.61, P>0.05, Table 5).

DNA products from stool samples were sequenced and validated as consistent with the *yst* gene by Shanghai Sangong Biological Engineering & Technology and Service Co. Ltd.

Table 1 Basic statistical analysis of the study sample

	Males:females	Children:adults	Citizens:farmers
Total number of samples	1,540:1,060	1,446:1,154	1,466:1,134
Positives	106:82	128:60	84:104
Incidence	6.9%:7.7%	8.9%:5.2%	5.7%:9.2%
Statistical analysis	u=0.86, P>0.05, no significance	u=3.52, P<0.001, significance	u=3.31, P<0.001, significance

The morbidity (from May 2005 to January 2008) was not significant between males and females, whereas it was significant between children and adults, as well as between farmers and citizens

Table 2 Incidence of yersiniosis in the four seasons

	Total samples	Positive (%)	Children	Adults
Spring	469	25 (5.3)	15	10
Summer	500	20 (4)	10	10
Autumn	775	58 (7.5)	41	17
Winter	856	85 (9.9)	62	23
Total	2,600	188 (7.23)	128	60

In Guangzhou, the distribution of the four seasons is as follows: spring from March to May, summer from June to August, autumn from September to November, and winter from December to February. Significance was noted in the morbidity(from May 2005 to January 2008) between autumn-winter and spring-summer months by the Mann-Whitney *U*-test (u=3.97, P<0.001), while no significance was noted between the autumn and winter months. Additionally, the constituent ratio of children and adults (with yersiniosis) in the four seasons was not significant by the Chi-square test (χ^2=5.07, P>0.05)

Bio/serotypes of virulent *Y. enterocolitica*

The bio/serotypes classification of 160 *Y. enterocolitica* strains examined by biochemical tests and diagnostic serum were as follows: 1A/unknown (four strains), 1B/unknown (eight), 2/O:9 (39), 2/unknown (seven), 3/O:3 (22), 3/unknown (six), 4/O:3 (55), 4/unknown (ten), and 5/unknown (nine). Due to the lack of a greater number of diagnostic serotypes, the other virulent strains (non-O:3 and O:9) were not confirmed.

Stool and blood examination

Eighteen positive stool samples were found with other bacteria or virus besides pathogenic *Y. enterocolitica*, including enteropathogenic *Escherichia coli* (two), *Campylobacter jejuni* (two), *Bacillus ceylonensis A* (three), *Paratyphosus A bacillus* (two), *Pseudomonas aeruginosa* (one), and rotavirus (eight).

Blood culture was performed in all nine septic patients caused by yersiniosis. Only five patients were positive for pathogenic *Y. enterocolitica*, whereas two patients negative for pathogenic *Y. enterocolitica* were positive for enteropathogenic *E. coli*.

Table 3 Distribution of incidence in different age groups

Age group (years)	Total samples	Positive(%)
0–1 (babyhood)	326	40 (12.3)
1–3 (infancy)	415	43 (10.4)
3–7 (pre-school age)	346	20 (5.8)
7–14 (school age)	202	16 (7.9)
14–18 (adolescence)	157	9 (5.7)
18–60 (adulthood)	544	17 (3.1)
Over 60 (senectitude)	610	43 (7.0)
Total	2,600	188 (7.23)

The morbidity of yersiniosis in children under 3 years of age was significantly higher than in children aged from 3 to 18 years by the Mann-Whitney *U*-test (u=3.26, P<0.05) and the incidence in adults over 60 years of age was statistically higher than in adults aged from 18 to 60 years by the Mann-Whitney *U*-test (u=3.0, P<0.05). In the high-risk group, the incidence of yersiniosis in children under 3 years of age was significantly higher than in adults over 60 years of age by the Mann-Whitney *U*-test (u=2.6, P<0.05)

Table 4 Incidence of yersiniosis in the three hospitals of this study

Hospital	Total	Positive(%)
Nanfang Hospital	1,600	126 (7.88)
Zhujiang Hospital	500	27 (5.4)
Guangdong Provincial People's Hospital	500	35 (7)
Total	2,600	188 (7.23)

The incidence of yersiniosis among the three hospitals (from May 2005 to January 2008) was not significant by the Chi-square test (χ^2=5.2, P>0.05). The three hospitals located in Guangzhou are affiliated to the Southern Medical University

Infection sources

The constituent ratio of children versus adults in infection sources did not show a significant difference by the Chi-square test (Table 6, χ^2=3.00, P>0.05). One hundred and one positive patients ingested suspicious food less than 3 days before the symptoms occurred, among whom, 45 patients confirmed that their friends sharing the same food manifested diarrhea while the others were not certain. We were unable to collect the samples of the patients' friends for detection, so it was unknown whether they were infected with virulent *Y. enterocolitica* or not. Forty-five patients who did not ingest suspicious food for certain had pets that may have been the infection sources.

Clinical manifestation

The clinical manifestation of yersiniosis mainly presented as gastroenteritis (156), pseudoappendicitis (32), and extra-intestinal complications (46). The incidence of extraintestinal complications in adults was significantly higher than

Table 5 Detection rate of the two methods by the McNemer test

RT-PCR	Culture method		Total
	+	−	
+	150	28	178
−	10	2,412	2,422
Total	160	2,440	2,600

The detection rate of RT-PCR was significantly higher than culture method by the McNemer test (χ^2=7.61, P>0.05)

Table 6 Distribution of infection sources among 188 positive patients

Infection sources	Children, *n*=128 (%)	Adults, *n*=60 (%)
Food	67 (52.3)	34 (56.7)
Beef	20	17
Chicken	25	14
Dog	14	10
Milk	25	7
Pork	50	21
Tap water	30	26
Vegetables	10	6
Unboiled water	25	20
Unknown food	10	8
Pets	35 (27.3)	10 (17.7)
Cat	20	3
Dog	15	7
Unknown reasons	26 (20.3)	16 (26.7)

The constituent ratio of children and adults in infection sources did not show a significant difference by the Chi-square test (χ^2=3.00, P>0.05). All data were collected from the patients when they visited the doctors. Some patients had ingested several kinds of foods. All foods were ingested (mainly in restaurants or from vendors in the street) less than 3 days before the symptoms occurred. Unknown food meant something ingested by patients who forgot about them, such as fruit. Patients who were ascribed to "Pets" were sure that they did not eat any suspicious food within 1 week, but all of them had pets. Unknown reasons mean patients did not have any suspicious food within 1 week and all of them had no pets

Table 7 Classification of clinical manifestation in 188 positive patients

Clinical manifestation	Children, *n*=128 (%)	Adults, *n*=60 (%)
Gastroenteritis	104 (81.3)	52 (86.7)
Abdominal pain	32	16
Bloody stool	67	30
Diarrhea	104	52
Fever	78	38
Nausea	15	5
Vomit	25	21
Pseudoappendicitis	24 (18.7)	8 (13.3)
*Extraintestinal complications	18 (14.1)	28 (46.7)
Arthritis	2	5
Erythema nodosum	2	5
Infection of the lung(s)	3	4
Myocarditis	1	2
Nephritis	5	4
Osteomyelitis	2	2
Septicemia	3	6

Significant difference was noted in the incidence of extraintestinal complications for children versus adults by the Mann-Whitney , U-test (u=4.85, , P<0.05) and it was the same for high-risk patients between adults over 60 (20/43) and children under 3 years of age (10/70, u=3.9, P<0.05). Each of the 46 patients with extraintestinal complications acquired only one disease belonging to extraintestinal complications. All complications* were diagnosed by professors according to disease guidelines

in the children by the Mann-Whitney U-test (u=4.85, P<0.05, Table 7) and it was the same for high-risk patients between adults over 60 (46.5%, 20/43) and children under 3 years of age (13.7%, 10/73, u=3.9, P<0.05). Additionally, there was no significance in the constituent ratio of children versus adults with yersiniosis in different systems by the Chi-square test (χ^2=1.5, P>0.05, Table 8).

A notable characterization of 32 patients diagnosed with pseudoappendicitis (24+8) was abdominal pain in the right lower quadrant, especially in or near the site of McBurney. Of 32 patients, 15 were performed with emergency operations, indicating nine cases of normal appendices, six of appendices with mild infiltration with inflammatory cells, 15 of terminal ileitis, and ten of mesenteric lymphadenopathy.

The manifestation of reactive arthritis in seven patients (male:female=2:5) mainly focused on pain, engorgement, and effusion in articular capsule, which involved the knee joint (four), ankle joint (three), articulationes digitorum pedis (two), articulationes digitorum manus (three), and wrist joint (three). Culture for virulent *Y. enterocolitica* with articular effusion was positive in only four patients. Reactive arthritis usually concomitantly occurred after a 1-week existence of gastrointestinal symptoms.

Erythema nodosum in seven patients (male:female=1:6) mainly happened on the anterior and posterior portions of the lower legs (five) and on the anterior portions of forearms (two). There was tenderness (seven), gargalesthesia (seven), and burning sensation (seven) in the red pars tuberalis, which become plaque within several weeks later. Five patients presented as erythema nodosum after 7 to 10 days existence of gastrointestinal symptoms, while two patients manifested both at the same time.

Of 46 patients with extraintestinal complications, nine were diagnosed with septicemia according to blood culture and clinical manifestation. Detailed information on these nine septic patients are listed in Table 9.

Distribution of family income among 188 positive patients

The constituent ratio of children and adults in the four levels of family income was not significant by the Chi-square test (χ^2=1.69, P>0.05, Table 10), but low-income families (under $300/month) occupied the majority in both children (75%) and adults (68%).

Antimicrobial susceptibility tests

All 160 virulent isolates from culture method were performed with antimicrobial susceptibility tests (Table 11), indicating TMP-SMX (99%, 158), Tobramycin (99%, 158),

Table 8 Distribution of 188 positive patients in each system

Underlying diseases in patients	Children, *n*=128 (%)	Adults, *n*=60 (%)
Diseases of the respiratory system	15 (11.7)	7 (11.7)
Chronic respiratory failure	0	1
Fungal infection of the lung(s)	5	2
Lung abscess	4	1
Lung cancer	0	1
Pulmonary tuberculosis	3	1
Pneumonia	3	0
Diseases of the circulatory system	6 (4.7)	4 (6.7)
Coronary heart disease	0	1
Heart failure	0	1
Infective endocarditis	1	1
Myocarditis	2	1
Valvular heart disease	3	0
Diseases of the digestive system	23 (18.0)	10 (16.7)
Cirrhosis of liver	0	2
Carcinoma of large intestine	0	1
Chronic viral hepatitis	7	0
Crohn's disease	4	1
Esophageal carcinoma	0	1
Intestinal tuberculosis	5	1
Primary carcinoma of liver	0	2
Tuberculosis peritonitis	4	1
Ulcerative colitis	3	1
Diseases of the urinary system	17 (13.3)	6 (10)
Acute glomerulonephritis	4	1
Chronic glomerulonephritis	2	0
Chronic renal failure	0	2
Nephrotic syndrome	4	2
Rapidly progressive glomerulonephritis	3	0
Tumor of the kidney(s)	4	1
Diseases of the hematological system	25 (19.5)	10 (16.7)
Aplastic anemia	3	1
Hemolytic anemia	3	1
Leukemia	4	2
Lymphoma	4	2
Iron deficiency anemia	2	1
Megaloblastic anemia	2	0
Malignant histocytosis	2	1
Sideroblastic anemia	5	2
Diseases of the endocrine system	9 (7.0)	5 (8.3)
Cushing syndrome	2	1
Diabetes mellitus	5	4
Graves disease	2	0
Rheumatic disease	2 (1.6)	1 (1.7)
Systemic lupus erythematosus	2	1
Diseases of bone and joint	5 (3.9)	3 (5)
Bone tuberculosis	2	1
Suppurative arthritis	1	1
Suppurative osteomyelitis	2	1
Unknown diseases (outpatients)	26 (20)	14 (23.3)

The constituent ratio of children versus adults with yersiniosis in different systems was not significant by the Chi-square test (χ^2=1.5, P>0.05). All diseases were diagnosed by professors in each system according to the guidelines of each disease

Gentamicin (99%, 158), Cefotaxime (98%, 157), Ceftazidime (94%, 150), Cefuroxime (82%, 131), Cefamandole (80%, 128), Ciprofloxacin (99%, 158), Ofloxacin (99%, 158), Ticarcillin/potassium clavulanate (8%, 13), Ampicillin (4%, 6), Cefazolin (9%, 14), and Cefradine (13%, 21). No multiple-resistant strain was found among the 160 isolates.

Discussion and conclusion

In this study, we carried out an epidemiological investigation of *Y. enterocolitica* from diarrheal patients with relatively sufficient number samples, in the hope of providing valuable clinical information on yersiniosis for clinicians. In addition, the reliability of RT-PCR was also investigated once more with a large number of samples.

Due to the disadvantages of culture methods, RT-PCR was introduced and had been primarily verified as a reliable and efficient method [18, 19]. Hence, it was once more used to detect another 1,900 clinical samples for epidemiology besides culture method. Of the total of 188 positive samples, 178 were confirmed by RT-PCR, while 160 were identified by culture method, indicating that the detection rate of RT-PCR was significantly higher than culture method (Table 5), which was contrary to our previous conclusion [18, 19]. This is possibly because stool samples were not sufficient enough to draw an objective conclusion in previous studies. As summarized in Table 5, ten positive samples confirmed by culture method could not be identified by RT-PCR, while 28 samples positive in RT-PCR were negative in culture method. Proverbially, PCR inhibitors found in tissues and feces can have significant adverse effects on the efficiency and sensitivity of PCR-based assays [22]. Therefore, these ten negative samples in RT-PCR might contain inhibitors that prevented the molecular reaction. On the other hand, 28 negative samples in culture method statistically showed its lower sensitivity than RT-PCR. In summary, although significance exists between the two methods, both RT-PCR and culture method, complementary to each other, are recommended to be used simultaneously in the clinical laboratory to increase the detection rate, which will be helpful to physicians for the diagnosis of patients.

From 1985 to 1987, an epidemiological investigation [9] of *Y. enterocolitica* was carried out with another culture method in China from diarrheal stools using the same diagnostic standards as the present research. The incidence was about 0.65% (256/39,412; details of this investigation have not been described in the literature), but now, the incidence in our final study was about 7.23%, which was significant. The reasons for this has been discussed in the former study [19]. Another study [23] in 2004 reported the

Table 9 Characteristics of nine septic patients

Age (years)	Sex	Bio/serotype	Mode of onset	Underlying disease	Clinical manifestation	Blood WBC (10^9/L)	Treatment	Outcome
3	Male	2/O:9	Acute	Intestinal tuberculosis	Gastroenteritis Hepatomegalia Jaundice	18.5	Gentamicin	Recovered
8	Male	4/O:3	Acute	Leukemia	Gastroenteritis Hepatomegalia Splenomegaly	16.8	Ciprofloxacin	Recovered
14	Female	3/O:3	Subacute	Aplastic anemia	Pseudoappendicitis Hepatomegalia	20.3	Cefotaxime	Recovered
55	Male	2/unknown	Acute	Lung abscess	Gastroenteritis Hepatomegalia Splenomegaly Jaundice	21.5	Cefotaxime	Recovered
68	Male	4/O:3	Subacute	Cirrhosis of the liver	Pseudoappendicitis Hepatomegalia Splenomegaly Jaundice	15.4	Ofloxacin Gentamicin	Recovered
48	Male	5/unknown	Acute	Primary carcinoma of the liver	Gastroenteritis Hepatomegalia Splenomegaly	16.4	Ciprofloxacin Tobramycin	Recovered
73	Female	1A/unknown	Acute	Carcinoma of the large intestine	Gastroenteritis Hepatomegalia Jaundice	15.5	Ceftazidime	Recovered
25	Male	2/O:9	Acute	Sideroblastic anemia	Pseudoappendicitis Hepatomegalia Splenomegaly	20.6	Ceftazidime	Recovered
67	Female	4/O:3	Subacute	Diabetes mellitus	Gastroenteritis Hepatomegalia Splenomegaly	17.9	TMP-SMX Cefotaxime Ampicillin	Died

Fever (>38°C) was found in all nine septic patients
TMP-SMX=trimethoprim-sulfamethoxazole

Table 10 Investigation of family incomes among 188 positive patients

Family income/month	Children, *n*=128 (%)	Adults, *n*=60 (%)
Less than USD 100	58 (45.3)	26 (43.3)
From USD 100 to USD 300	38 (29.7)	15 (25)
From USD 300 to USD 500	22 (17.2)	11 (18.3)
Over USD 500	10 (7.8)	8 (13.3)

The family income was offered by the patients or their parents when they visited the doctors. The constituent ratios of children and adults in the four levels of family income was not significant by the Chi-square test (χ^2=1.69, P>0.05). Low-income families (under \$300/month) occupied the majority in both children and adults

incidence of yersiniosis (9.8%, 16/182) in China by culture methods, which was not significantly discrepant with our study by the Mann-Whitney *U*-test (u=1.35, P>0.05).

Theoretically, *Y. enterocolitica*, transmitted mainly by an oral–fecal route [24, 25], can survive from −2 to 45°C, and its most suitable growth temperature is from 28 to 29°C [9], so the cooler autumn-winter months in Guangzhou are more suitable for growth than the warmer spring-summer months. In this study, significance was noted in the morbidity between autumn-winter and spring-summer months (Table 2), while no significance was noted between the autumn and winter months (Table 2), which was a little different from the USA [26]. Noticeably, other diarrheal diseases, such as rotaviral enteritis, may also focus on autumn-winter months, increasing the difficulty in the diagnosis for clinicians. Even so, as a doctor, relatively rare diarrhea diseases, such as yersiniosis and *Campylobacter jejuni* enteritis, should be taken into consideration when the final diagnosis is obscure.

A high morbidity in children under 4 years of age was reported [9], but the incidence in adults over 60 years of age, a high-risk group, was seldom described. Moreover, little information is available for the comparison of the incidence between children under 3 years of age and children aged from 3 to 18 years, as well as between adults over 60 years of age and adults aged from 18 to 60 years. In this study, the questions above were answered preliminarily. According to the statistical data (Table 3), children under 3 and adults over 60 years of age are much more easily infected by *Y. enterocolitica* than other age groups, probably on account of their incomplete immunological function and/or underlying diseases.

The serotypes of *Y. enterocolitica*, such as 2/O:9 (39), 3/O:3 (22), and 4/O:3 (55), isolated from 160 positive patients were mainly pathogenic serotypes in China [9]. In previous studies [18, 19], we reported mixed infections of serotypes O:3 and O:9. However, we found that it was a mistake by the technician, so no mixed infection was found in previous studies and this study. Although mixed

Table 11 Antimicrobial susceptibility tests of the 160 isolates

Bio/serogroup (number)	TMP-SMX (99%, 158)	Tobramycin (99%, 158)	Gentamicin (99%, 158)	Cefotaxime (98%, 157)	Ceftazidime (94%, 150)	Cefuroxime (82%, 131)	Cefamandole (80%, 128)	Ciprofloxacin (99%, 158)	Ofloxacin (99%, 158)	Ticarcillin/potassium clavulanate (8%, 13)	Ampicillin (4%, 6)	Cefazolin (9%, 14)	Cefradine (13%, 21)
1A/unknown (4)	+	+	+	+	1 (−)	1 (−)	2 (−)	+	+	−	−	−	2 (+)
1B/unknown (8)	+	+	1 (−)	1 (−)	+	1 (−)	2 (−)	+	+	−	−	1 (+)	2 (+)
2/O:9 (39)	1 (−)	+	+	+	1 (−)	8 (−)	6 (−)	1 (−)	+	4 (+)	2 (+)	1 (+)	4 (+)
2/unknown (7)	+	+	1 (−)	+	1 (−)	3 (−)	2 (−)	+	1 (−)	−	1 (+)	2 (+)	−
3/O:3 (22)	+	+	+	1 (−)	1 (−)	4 (−)	6 (−)	+	+	3 (+)	2 (+)	2 (+)	3 (+)
3/unknown (6)	1 (−)	+	+	1 (−)	+	1 (−)	3 (−)	+	+	−	−	1 (+)	1 (+)
4/O:3 (55)	+	1 (−)	+	+	2 (−)	8 (−)	6 (−)	+	1 (−)	4 (+)	1 (+)	3 (+)	4 (+)
4/unknown (10)	+	+	+	+	2 (−)	2 (−)	3 (−)	1 (−)	+	1 (+)	−	2 (+)	2 (+)
5/unknown (9)	+	1 (−)	+	+	2 (−)	1 (−)	2 (−)	+	+	1 (+)	−	2 (+)	3 (+)

TMP-SMX=trimethoprim-sulfamethoxazole; +=positive for all; −=negative for all; 1 (−)=one negative, the other positive; 1 (+)=one positive, the other negative

infections did not exist in our research, it has been confirmed in some outbreaks [9]. Serotype O:9 was reported [9] to correlate with HLA-27, which caused arthritis in patients. Five patients infected with O:9 were diagnosed as arthritis, but two patients infected with other serotypes were also diagnosed with arthritis, which was not found in a previous study [19]. Although not all patients infected with O:9 would develop arthritis, we should pay more attention to arthritis when infected with O:9. Remarkably, four strains isolated from four patients were confirmed to be biogroup 1A, among which, three were also identified with the *yst* gene by RT-PCR. Coincidently, routine stool examination found no other pathogen in these four samples. Biogroup 1A was generally regarded as nonpathogenic, but several studies reported that biogroup 1A had been isolated from humans with clinical yersiniosis [27, 28], which was also verified in this research. Additionally, biogroup 1A strain, causing septicemia in a 73-year-old patient with liver cancer, may be firstly described by us (Table 11.). Therefore, some of the biogroup 1A strains, although lacking traditional virulence markers, are able to cause yersiniosis and fatal complications in humans with unknown virulence genes in chromosome and plasmid.

It is commonly known that diarrheal patients may contain two or more pathogenic bacteria in stool samples. In this research, 18 positive samples were found with other bacteria or virus besides pathogenic *Y. enterocolitica* by routine stool examination. However, one question arises that *Y. enterocolitica* is not involved in the scope of routine stool examination in most of hospitals and even that detection for *Y. enterocolitica* does not exist in some hospitals. Because of this, if diarrheal patients are infected with *Y. enterocolitica* and *Staphylococcus aureus*, for example, clinicians cannot form a correct diagnosis according to the routine stool examination and then choose the susceptible antibiotics. Furthermore, it is impossible for doctors to diagnose patients as yersiniosis only by clinical manifestation, which is similar in most of the diarrheal diseases. Therefore, all diarrheal stool samples need to be performed with detection for *Y. enterocolitica*. Finally, detection for *Y. enterocolitica*, a gold standard for diagnosis, should be brought into the scope of routine stool examination.

Y. enterocolitica can survive from −2 to 45°C, so it can exist in refrigerators and water, causing yersiniosis easily. Pig, cattle, and chicken are healthy carriers of virulent *Y. enterocolitica*, which are the main food consumption group for people in daily life [9]. From Table 6, foods occupied about 54% of infection sources, while pets occupied about 24%. Although the constituent ratio of children and adults in infection sources did not show a significant difference, we can see that foods were the main infection sources in both groups. According to the patients, all of the foods (Table 6) may not have been fully cooked before being ingested. Due to thirst, tap water and unboiled water were drunk mainly by children, workers, and farmers in their convenience. In the past, little information on vegetables as an infection source was reported [29, 30]. The direct isolation of virulent *Y. enterocolitica* from vegetables was not available in this study, but all 16 patients recalled that they had raw vegetables with other boiled meats. Hence, we were highly suspicious of the raw vegetables. However, further identification is needed in our future study. On the other hand, 45 patients who were sure that they did not eat any suspicious foods within 1 week of the symptoms might probably have been infected from their pets. Pet animals that are healthy carriers of virulent *Y. enterocolitica* have been suspected as being sources of human yersiniosis because of their close contact with humans, especially young children [30]. Furthermore, it is reported that [30] raw pork should not be given to pets because pathogenic *Y. enterocolitica* can be easily transmitted from highly contaminated raw pork to pets. Dogs and cats may be an important transmission link of pathogenic *Y. enterocolitica* between pigs and young children. Unfortunately, in this study, most of the 45 patients often fed their pets with raw meats, such as pork, beef, and fish, which may explain the origin of infection and the association between *Y. enterocolitica* and patients. Referring to the results above, we can prevent infection of yersiniosis by extensively educating children, workers, and farmers that meat, especially pork and beef, must be fully cooked before ingestion, while tap water and unboiled water must not be drunk under any circumstances. In addition, close contact with pets is not recommended in order to reduce the potential risk of infection.

The clinical manifestation of yersiniosis in 188 positive patients mainly presented as gastroenteritis (156, 83%) and pseudoappendicitis (32, 17%). Extraintestinal complications occurred in 46 (24%) of 188 patients. All 156 patients belonging to the gastroenteritis group manifested fever (62%), diarrhea (100%), blood stool (52%), abdominal pain (26%), vomit (24%), and nausea (11%). In all 104 children with gastroenteritis, 82% of them were under 5 years of age, indicating that *Y. enterocolitica* infection in children under the age of 5 years mostly manifested gastroenteritis.

The notable characterization of pseudoappendicitis was abdominal pain in the right lower quadrant, especially in or near the site of McBurney, in addition to gastrointestinal symptoms. Owing to this fact, it is very easy to make a wrong diagnosis of acute appendicitis by emergency doctors. Differences of clinical manifestation between pseudoappendicitis and acute appendicitis, as well as other diarrheal diseases, are impractical for differentiating emergent patients. However, pseudoappendicitis is often caused

by unboiled food, especially pork containing *Y. enterocolitica*, while acute appendicitis is seldom by this reason. Hence, etiological factors may be helpful to clinicians. Further pathologic diagnosis in 15 patients with emergency operations indicated that the diseased region mainly focused on terminal ileum and mesenteric lymph nodes, explaining why the position of abdominal pain was located in or near the site of McBurney. Additionally, in all 24 children with pseudoappendicitis, 67% of them were over 5 years of age, revealing that pseudoappendicitis is the main clinical manifestation of yersiniosis in children over the age of 5 years.

Extraintestinal complications were found in 18 children and 28 adults. Ten of 18 children with extraintestinal complications were under 3 years of age, accounting for 56%, while 20 of 28 adults were over 60 years of age, occupying 71%, which hinted that the high-risk group was easily involved in complications. The incidence of extraintestinal complications in adults was significantly higher than in the children (Table 7), while the morbidity of extraintestinal complications in adults over 60 years of age (20/43) was statistically higher than in children under 3 years of age (10/73). The reason for this might be correlated with different underlying diseases and infected bio/serotypes in adults and children besides their constitution and immunological function. It is reported that [9] patients with liver cirrhosis, nephritis, and diabetes mellitus are prone to contract yersiniosis and a relevant extraintestinal infection, such as septicemia.

All nine septic patients were derived from different underlying diseases (Table 9), including liver diseases, tumor, hematopathy, and tuberculosis, which could cause a lower power of resistance due to chemical therapy. Hence, *Y. enterocolitica* could easily enter the blood through intestinal mucosa, leading to bacteremia or even septicemia. Iron is an essential factor for the growth of bacteria [31]. *Y. enterocolitica* bacteremia and other systemic infections are more often seen in patients with iron overload and those receiving chelation therapy [32]. In this study, one septic patient with sideroblastic anemia was identified. All nine septic patients receiving susceptible antibiotics treatment recovered within 2–3 weeks, except for one case who died. Owing to the lack of knowledge and vigilance for complications of yersiniosis, susceptible antibiotics could not be used in this patient at an early stage, so the patient deceased. Moreover, in septic patients caused by *Y. enterocolitica*, antibiotics treatment (with supportive treatment) cannot be stopped until some time (3–5 days recommended) after negative results in both blood and stool, as well as the resolution of symptoms, which is critical in order to save the patients.

The concentration of pathogenic *Y. enterocolitica* in 188 positive stool samples ranged from 10^5 to 10^8 CFU/g detected by RT-PCR for the first time. Among 40 outpatients, no *Y. enterocolitica* was found after antibiotics treatment for 1 week. One month later, eight of 40 outpatients returned for a followup visit, showing no gastrointestinal symptoms, but two patients were positive by RT-PCR (10^3 CFU/g). In fact, no symptom carriers in human are likely to become the infection source [9]. Therefore, these two patients were administered with antibiotics for another week and none were found at the end of the treatment. All 40 outpatients without wasting diseases entirely recovered. On the other hand, among 148 inpatients, there was no *Y. enterocolitica* in 102 patients after antibiotics treatment for 1 week, while there was still *Y. enterocolitica* with concentrations from 10^3 to 10^6 CFU/g in 46 patients who developed complications later. So, these 46 patients continued the treatment and, until 2 to 3 weeks later, no *Y. enterocolitica* was found in their stools by both methods. After 3 weeks, ten samples of 102 inpatients without complications were collected and one inpatient was positive by both methods, who had to continue with antibiotics treatment for another week. Finally, no *Y. enterocolitica* was found in this patient after treatment. In relation to prognosis, 102 inpatients without complications fully recovered, while 46 inpatients with complications were partly rehabilitated without gastroenteritis 2 weeks later, but lived with extraintestinal infection for about a month, such as arthritis and erythema nodosum. Furthermore, owing to the lowest detection limit at about 10^3 CFU/g by RT-PCR, it is possible that *Y. enterocolitica,* in low concentration still exists in the stool samples of some patients after treatment, which cannot be detected. Thus, doctors are easily misled into stopping antibiotics treatment prematurely. In conclusion, the early diagnosis, treatment, and precautions for complication are critical for yersiniosis, while early treatment with susceptible antibiotics should be extensively advocated and antibiotics treatment should not be stopped until some time (3–5 days recommended) after negative results in stools in order to kill the residual *Y. enterocolitica* which are probably still present in low concentration.

In Table 8, we found that 148 inpatients were mainly administrated in departments of respiration (11.7%), gastroenterology (18%), nephrology (13.3%), and hematology (19.5%). The diseases listed are so serious and exhausting that they can easily lead to bacterial infection in patients, especially in adults over 60 (72%, 43/60) and children under 3 years of age (65%, 83/128). Crohn's disease (CD) and ulcerative colitis (UC) with *Y. enterocolitica* infection were reported [33], which was also confirmed by us. In this research, five positive samples were found in 37 CD patients, while four were found in 39 UC patients, the incidence of which accorded with Saebo et al. [33], but differed from Lamps et al. [34]. However, it is impossible

to distinguish whether these patients had an original presentation of CD or UC with concomitant yersiniosis or primary *Y. enterocolitica* infection followed by separated onset of CD or UC. Some investigators who followed patients with acute ileal yersiniosis for years found that none of them developed evidence of CD [35]. As far as clinicians are concerned, routine stool examination is needed when inflammatory bowel disease patients occurred with continuous diarrhea, which can help us exclude bacteria infection, such as *Y. enterocolitica*, and choose antibiotics according to susceptibility tests.

Drug susceptibility testing is important for doctors in order to choose susceptible antibiotics. All clinicians in this study regulated antibiotics according to the susceptibility tests, which avoided the abusage of antibiotics and the occurrence of multiple-resistant strains. Antimicrobial susceptibility tests (Table 11) showed that the third-generation cephalosporins, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole were effective in the treatment of yersiniosis and its complications, such as septicemia, whereas the first-generation cephalosporins and penicillins were not. Based on this data, clinicians can choose the susceptible antibiotics above when yersiniosis is identified and then adjust the antibiotics treatment by referring to the susceptibility test results.

In the past, the traditional culture method was a fundamental tool for detecting *Y. enterocolitica* from stool samples, which was time-consuming and less efficient, possibly contributing to a low incidence of yersiniosis. Therefore, epidemiological investigation only by the culture method may be unreliable and biased. In this study, an epidemiological investigation of yersiniosis by both RT-PCR and improved culture method was carried out with relatively sufficient number of 2,600 samples. As a result, RT-PCR, with a higher detection rate than culture method, was once again validated as an efficient tool for the detection of virulent *Y. enterocolitica*. On the other hand, based on results in this study, *Y. enterocolitica* is a food-borne pathogen causing clinical manifestation, such as gastroenteritis, pseudoappendicitis, and extraintestinal complications. During autumn-winter months, it is necessary for clinicians to extensively publicize the general knowledge of hygiene and medicine in rural areas for children, farmers, and workers in order to prevent infection and outbreak, and to pay more attention to the early diagnosis of yersiniosis and its complications through stool and blood examination, as well as clinical manifestations, aiming at children under 3 and adults over 60 years of age with wasting diseases. Moreover, early or prophylactic treatment with susceptible antibiotics, including third-generation cephalosporins, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole, should be advocated for suspicious patients with yersiniosis and then adjusted according to the susceptibility test results. Moreover, antibiotics treatment should not be stopped until some time (3–5 days recommended) after negative results in both stool and blood.

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