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Diagnosis of Yersiniosis

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Foreword

This standard is drafted in accordance with the rules given by GB/T 1.1-2009.

This standard is centralized by the Chinese Preventive Medicine Association.

The drafting agencies of this standard are National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention; Chinese Center for Disease Control and Prevention; Beijing Ditan Hospital Capital Medical University; Peking University People's Hospital; Jiangsu Provincial Center for Disease Control and Prevention; Zhengzhou Engineering Research Center for Rapid Detection Reagent of Food-borne Pathogens; Subei Mongolian Autonomous County Center for Disease Control and Prevention; China National Center for Food Safety Risk Assessment.

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Diagnosis of Yersiniosis

1 Scope

This standard provides the diagnostic basis, diagnostic principles, diagnosis and differential diagnosis of yersiniosis.

This standard is applicable to the pathogen detection, diagnosis and report of yersiniosis by the staff of medical institutions and Centers for Disease Control and Prevention at all levels in China.

2 Terminologies and Definitions

The following terminologies and definitions apply to this document.

2.1

Yersinia

A bacterium of the genus *Yersinia*, family Enterobacteriaceae. To date, 18 species have been identified, among which *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis* are pathogenic to humans.

2.2

Yersiniosis

Yersiniosis is mainly caused by infection of *Y. enterocolitica*, and a few cases are caused by *Y. pseudotuberculosis*, both of which cause zoonotic, foodborne and gastrointestinal infectious diseases, with diarrhea as the main clinical manifestation. A few cases may present with erythema

nodosum, reactive arthritis, and even sepsis and other extraintestinal complications.

Note: the disease caused by *Yersinia pestis* that infects humans is called plague.

3 Diagnostic basis

3.1 Epidemiological history

Yersiniosis can occur all year round and often in cold seasons. The main sources of infection are swine and canine. Before onset, most patients have consumed unclean food, especially food preserved by refrigeration; consumed unclean water; or had contact with yersiniosis patients, human carriers or animals carrying the pathogen. Blood transfusions from patients or carriers with *Y. enterocolitica* or *Y. pseudotuberculosis* can lead to bloodborne transmission of yersiniosis. Since food preserved in refrigerators is an important source of infection, this disease is also known as "refrigerator disease". Detailed epidemiological characteristics are shown in Appendix A.

3.2 Clinical manifestations

The clinical manifestations of yersiniosis are complex and varied, which can be divided into gastroenteritis-type, appendicitis-like-type (terminal ileitis-type), reactive arthritis-type, erythema nodosum-type and sepsis-type.

Gastroenteritis-type: This type is the most common presentation, manifesting mainly as acute diarrhea. The diarrhea frequency is from 3 ~ 5 times /day, or up to more than 10 times/day, and some cases may be accompanied by fever and abdominal pain. The feces trait changes include loose stools, mucus stools, or bloody pus stools.

Appendicitis-like-type: This presentation is the terminal ileitis type, which presents with obvious right lower abdominal pain, and some cases are clinically diagnosed as appendicitis.

A small number of patients can also develop extraintestinal complications, including mainly the following:

Reactive arthritis-type: This presentation is the most common type of extraintestinal yersiniosis, characterized by joint pain, swelling, and joint capsule fluid exudation.

Erythema nodosum-type: At 1 ~ 2 weeks after gastroenteritis, some patients may develop erythema nodosum or pleomorphic erythema.

Sepsis-type: Patients with bloodborne infection or immunodeficiency may develop sepsis, which is rare but has a high mortality rates.

Detailed clinical manifestations are shown in Appendix B.

3.3 Laboratory tests

3.3.1 Routine stool examination

The feces trait changes include mainly watery or loose stools, sometimes mucus stools, and occasionally blood or pus and blood in the feces. In most patients' stool microscopic examination, white blood cells have been found, and some may also present red blood cells.

3.3.2 Rapid test of sample

Colloidal gold-labeled *Y. enterocolitica*- or *Y. pseudotuberculosis*-specific antibody rapid detection reagent can directly detect the pathogens in patients' fresh stools and/or in enrichment broth of stools, anal swabs, blood (whole blood or blood clots) or tissue samples (mesenteric lymph nodes, removed appendix, endoscopy-obtained intestinal wall, etc.). See Appendix C for a detailed operation procedure.

3.3.3 Nucleic acid detection of samples

This diagnostic test is used to detect one or more positive genes among the *Y. enterocolitica* ferrioxamine receptor (*foxA*) or adhesion invasion locus (*ail*) or *Y. pseudotuberculosis* invasin (*inv*) genes from any patient stool, anal swab, blood (whole blood or blood clot), or tissue samples

(mesenteric lymph nodes, removed appendix, endoscopy-obtained intestinal wall, etc.), etc. See Appendix C for a detailed operation procedure.

3.3.4 Isolation and culture of pathogen

This diagnostic test is used to isolate *Y. enterocolitica* or *Y. pseudotuberculosis* from any patient stool, anal swab, blood (whole blood or blood clot), or tissue samples (mesenteric lymph nodes, removed appendix, endoscopy-obtained intestinal wall, etc.), etc. See Appendix C for a detailed operation procedure.

4 Diagnostic principles

Diagnosis is achieved according to epidemiological history, clinical manifestations and laboratory detection.

5 Diagnosis

5.1 Clinical diagnosis

A clinically diagnosed case meets one of the following conditions:

a) In accordance with 3.2, 3.3.1 and 3.3.2, 3.1 for reference.

b) In accordance with 3.2, 3.3.1 and 3.3.3, 3.1 for reference.

5.2 Confirmed cases

A confirmed case meets one of the following conditions:

a) In accordance with 3.2, 3.3.1 and 3.3.4.

b) In accordance with 3.2, 3.3.1 and 3.3.3, the PCR amplification products for one or more of the target genes *foxA*, *ail* and *inv* were sequenced, and the sequence is consistent with reference sequences in nucleic acid sequence databases, such as GenBank.

6 Differential diagnosis

6.1 Gastroenteritis-type yersiniosis should be differentiated from bacterial dysentery and diarrhea caused by enteroinvasive *Escherichia coli* and *Salmonella*.

6.2 Appendicitis-like-type (terminal ileitis-type) yersiniosis should be differentiated from acute and chronic appendicitis.

6.3 Reactive arthritis-type yersiniosis should be differentiated from septic arthritis caused by streptococci, staphylococci and other bacteria.

6.4 Erythema nodosum-type yersiniosis should be differentiated from drug-induced erythema and allergic erythema.

6.5 Sepsis-type yersiniosis should be differentiated from sepsis caused by other bacteria.

Appendix A
(informative appendix)

Yersiniosis Epidemiology

A.1 Epidemiological characteristics

Yersiniosis is a global disease that has spread across all continents. Europe is the region with the highest infection rate of *Y. enterocolitica*, where the main epidemic type is 4/O:3. Belgium, Finland, and Sweden are among the European countries with most cases. The most recent report was an outbreak in Sweden in the first half of 2018 due to the consumption of pig intestines. China had two outbreaks in the 1980s, and approximately 500 people were infected. According to surveillance from 2010 to 2015, the infection rate of *Y. enterocolitica* in China is approximately 0.59%, which is not lower than that in other countries. The prevalent bioserotype in China is mainly 3/O:3, which is different from that in Europe. It has been found that diarrhea caused by *Y. enterocolitica* in China is often diagnosed as bacterial dysentery, so the actual prevalence of *Y. enterocolitica* in China may be underestimated. The infection rate of *Y. pseudotuberculosis* is generally lower than that of *Y. enterocolitica*. *Y. pseudotuberculosis* is most distributed in the northern hemisphere. In the southern hemisphere, *Y. pseudotuberculosis* is found in mainly Australia and New Zealand and is rarely reported in South America (except Brazil) and Africa. Finland and Japan are the countries that reported the most infection cases of *Y. pseudotuberculosis*.

Yersinosis can be seen all year round but is more frequently found during the cold season because of the psychrophilic characteristic of *Yersinia*.

A.2 Source of infection

Y. enterocolitica and *Y. pseudotuberculosis* are both zoonotic pathogens and have a wide range of animal hosts, including humans and all warm-blooded wild or domestic animals. Pigs and dogs are the main hosts and sources of infection for *Y. enterocolitica*. Occasionally, *Y.*

enterocolitica has been found in reptiles, fish and shellfish. It has been confirmed that insects, such as flies and cockroaches, carry *Y. enterocolitica* and that *Y. enterocolitica* can be isolated from the external environment as well. Slaughtered pigs or undercooked pork and visceral products of pigs are the main sources of yersiniosis. According to a survey conducted in 11 provinces and cities in China from 2008 to 2010, the average positive rate of *Y. enterocolitica* in slaughtered pigs was 19.53% (878/4,495). Molecular epidemiological investigations have confirmed that dogs are also a major source of infection with *Y. enterocolitica*. Other domestic animals, rodents, birds, and other wild animals may also carry *Y. enterocolitica*. *Y. pseudotuberculosis* is more widely distributed than *Y. enterocolitica*, and the *Y. pseudotuberculosis* infection rate in birds is usually much higher than that of *Y. enterocolitica*. Migrant bird migration has played a significant role in the widespread dissemination of *Y. pseudotuberculosis* between different continents around the world.

A.3 Route of transmission

Y. enterocolitica and *Y. pseudotuberculosis* are transmitted through mainly the fecal-oral route. Ingestion of contaminated food and water is the main route leading to human infections, especially outbreaks. Contact with patient stool also leads to infection between people. Direct contact with the excrement of animal carriers can also cause infection.

Y. enterocolitica and *Y. pseudotuberculosis* are foodborne pathogens that can be transmitted through contaminated food stored in refrigerators. The contamination of these bacteria may occur in various processes, such as manufacturing and processing of food, cutting, dispensing, handling, and selling of unpackaged foods. *Y. enterocolitica* has been isolated from a variety of raw and cooked meat, vegetables, milk and dairy products, juice drinks and other foods. Due to the psychrophilic nature of the bacteria, it can survive and reproduce in food stored in a refrigerator for a long time. Contaminated food stored at low temperature in a refrigerator is an important source of yersiniosis. Epidemic spread through water or soil is due to mostly the contamination of water and soil by the feces of infected animals.

The injection of red blood cells contaminated with *Y. enterocolitica* or *Y. pseudotuberculosis* is one of the causes of blood transfusion-related infections. Since these bacteria can reproduce at

refrigerated temperatures, blood transfusion-related infections may also be caused by refrigerated blood. Although bloodborne transmission of yersiniosis is relatively rare, it still needs to be taken seriously because it usually leads to sepsis in patients, which has a high mortality rate.

A.4 Susceptible population

The population is generally susceptible, with a high incidence in infants and young children, who present with mostly diarrhea-type cases. The incidence of other clinical cases in children is lower than that in adults. The symptoms of older children and adolescents are mostly those of appendicitis-like-type yersiniosis. Immunodeficient patients, such as antigen HLA-B27 allele carriers; patients with liver disease, diabetes, blood diseases, or organ transplantation; and human immunodeficiency virus (HIV) carriers, have an increased likelihood to develop extraintestinal complications, such as reactive arthritis. Severe infections are generally observed in people who use immunosuppressive agents, have immunodeficiency or have iron overload.

Appendix B
(informative appendix)
Clinical Manifestations of Yersiniosis

B.1 Overview

After entering the human intestine, *Yersinia* invades the lower lymphoid tissue Peyer's knot through the intestinal mucosa. It grows and reproduces in the intestinal lumen and intestinal lymphoid tissues, causing a dramatic proliferation of multinucleated leukocytes, leading to acute inflammation. A few patients have the pathogens spread through the bloodstream. Due to the lymphotropic features of *Yersinia*, they may spread through the lymphatic vessels and reach the joints and other tissues, causing extraintestinal yersiniosis.

The incubation period of yersiniosis is 1 d to 10 d. The patient can excrete the bacteria for a long time, and the incubation period is long. Due to different strain types and individual health, reactivity, and immune levels, clinical manifestations are different: diarrhea is common in infants and young children; erythema nodosum is common in adults over 40 years old; mesenteric lymphadenitis occurs mostly in adolescents and older children; autoimmune phenomena is common in women; HLA-B27 patients are prone to arthritis; and systemic infections occur frequently in patients with iron overload.

B.2 Clinical stages

B.2.1 Acute stage

In this stage, acute inflammation is the main symptom, and the clinical manifestations vary depending on the infected organ. Pathogens can usually be isolated from the affected location. The symptoms include mainly gastroenteritis, lymphadenitis, terminal ileitis (pseudoappendicitis), pneumonia, sepsis, etc. Among them, gastroenteritis is most common and can occur in any age

group, but infants and young children under 5 years old have the highest incidence of this type.

B.2.2 Complication stage

This stage is manifested as mainly reactive arthritis, erythema nodosum, myocarditis, etc., and occurs from 1 week to 3 weeks after the acute stage. Most of the complications are serious and often require hospitalization.

B.2.3 Recurrence stage

The main symptoms are autoimmune diseases such as polymyositis, rheumatoid arthritis, lupus erythematosus, and nodular polyarthritis.

B.3 Clinical type

B.3.1 Gastroenteritis

Acute gastroenteritis is the most common clinical manifestation of yersiniosis, with typical symptoms of diarrhea and fever. Infants and young children have a high proportion of cases with such symptoms, and the severity varies. Diarrhea manifests as watery stools and mucous stools, and in severe cases, bloody stools can occur. The number of daily diarrhea events varies from 3-5 times/d to more than 10 times/d. White blood cells and/or red blood cells can be seen by routine stool test. At present, some clinical diarrhea caused by *Y. enterocolitica* is often diagnosed as bacterial dysentery in China.

B.3.2 Pseudoappendicitis (terminal ileitis)

Some cases show pain in the lower-third of the right abdomen, creating a clinical emergency often diagnosed as appendicitis, but the appendix is often found to be normal after appendectomy, while enlargement of the terminal ileum, appendix, and mesenteric lymph nodes is often observed in such cases.

B.3.3 Reactive arthritis

Reactive arthritis-type is the most common type of extraintestinal yersiniosis, which occurs

in mainly adults, who are mostly female. Local joint symptoms are mainly pain, swelling and exudation of joint capsule fluid.

B.3.4 Erythema nodosum

Erythema nodosum is another type of intestinal yersiniosis. Some adult yersiniosis cases may develop erythema nodosum or erythema multiforme 1 week to 2 weeks after gastroenteritis, but some studies have shown that 40% of cases lack gastrointestinal symptoms.

B.3.5 Sepsis

This type is uncommon, but the symptoms are severe, and the mortality rate is close to 50%. Infected patients with a history of cirrhosis, diabetes, malignant tumors, severe anemia, and blood diseases can develop sepsis, yet a small number of ordinary infected people may also experience this presentation.

Appendix C

(normative appendix)

Laboratory Testing for Yersiniosis

C.1 Collection, transfer and storage of Samples

Stool, rectal swab, blood (whole blood or clots) or tissue (mesenteric lymph nodes, resected appendix, intestinal wall, etc.) samples of diarrhea patients are collected for the isolation of *Y. enterocolitica* and *Y. pseudotuberculosis*. Upon stool trait changes, the pus or mucus part should be taken when collected.

The sample is divided into two parts immediately after collection: one is quickly transferred to a microbiology laboratory at 4°C in a sterile container for nucleic acid detection according to the method of C.3; and another is immediately inoculated into enrichment solution or on selective agar according to the method of C.4 and transferred to a microbiology laboratory within 24 h according to the corresponding temperature of enrichment and culture.

If inoculation of solution or medium cannot be performed when the sample is collected, >5 mL or >5 g of a sample can be temporarily placed in Carry-Blair transport medium at 4°C and transferred to the microbiology laboratory within 24 h to 48 h for nucleic acid detection or isolation and culture.

If the sample cannot be detected in time or needs to be stored for a long time, it is recommended to place the sample in 25% to 30% sterilized glycerin broth and immediately freeze the sample at ≤ -20°C. The storage and transfer temperature should be kept at ≤ -20°C. The strain can still be isolated after 3 to 6 months of storage, but the isolation efficiency may be affected, but the efficiency of nucleic acid detection will not be greatly affected.

C.2 Rapid detection of samples

C.2.1 Rapid test solution

Taking a small amount of sample, stool, rectal swab, blood (whole blood or blood clots), mesenteric lymph node, resected appendix or endoscopically obtained intestinal wall, in 1 mL of a saline solution, the sample is shaken well and mixed; after natural sedimentation, the supernatant is taken as the rapid test sample solution. For enrichment sample, the sample is mixed by inversion in an enrichment tube, and the supernatant is taken as the rapid test solution after natural sedimentation.

C.2.2 Detection method

When the rapid test solution is prepared, a colloidal gold test card is opened, and 1 to 2 drops (approximately 100 μL to 150 μL) of the rapid test solution is added to the sample well on the test card. It must be ensured that no bubbles exist during the procedure. The timer is set, and the result is determined within 15 min to 20 min after the sample is added.

C.2.3 Results interpretation

C.2.3.1 Positive result

Both the quality control line and detection line appear, suggesting a specific antigen is detected.

C.2.3.2 Negative result

The quality control line appears, but the detection line does not appear, suggesting no specific

antigen is detected.

C.2.3.3 Invalid result

If the quality control line does not appear, the test result will be invalid regardless of whether the detection line appears. The test should be repeated.

C.2.4 Limitations of the method

C.2.4.1 False-positive results

When the positive result is not consistent with the clinical manifestation, further confirmation should be performed by other methods.

C.2.4.2 False-negative results

A false-negative result occurs when the antigen concentration of the sample is below the test limit of detection. When the negative result is not consistent with the clinical manifestation, further confirmation should be performed by other methods.

C.3 Nucleic acid detection of samples

C.3.1 Detection method

C.3.1.1 qPCR method

qPCR amplification is performed in 20 μL reaction volumes: 10 μL of commercial Premix; 7.2 μL of ultrapure water; 0.2 μL of ROX; 0.2 μL of upstream primers, downstream primers and probes each (100 nmol/L each); and 2 μL of sample DNA. Primer and probe sequences are shown in Table C.1.

The reaction mixture is incubated at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

Table C.1 Primers and probes for qPCR of the *ail* and *foxA* genes

Primer/probe	Sequence/ 5′ → 3′	Amplification length/bp
ail-F	TTTGGAAGCGGGTTGAATTG	101
ail-R	GCTCACGGAAAGGTTAAGTCATCT	
ail probe	FAM-CTGCCCCGTATGCCATTGACGTCTTA-BHQ	
foxA-F	ACGGCGGTGATGTGAACAA	85
foxA-R	GGGTCCACTTGCAGCACATT	
foxA probe	FAM-ACCTTCCTTGATGGGCTGCGCTTACTC-BHQ	
IAC-F	GCAGCCACTGGTAACAGGAT	118
IAC-R	GCAGAGCGCAGATACCAAAT	
IAC probe	HEX-AGAGCGAGGTATGTAGGCGG-TAMRA	

C.3.1.2 Regular PCR method

Regular PCR amplification is performed in 20 μL reaction volumes: 10 μL of commercial Premix; 8 μL of ultrapure water; 0.5 μL of upstream and downstream primers each (10 μmol/L each); and 1 μL of sample DNA. Primer sequences are shown in Table C.2.

The reaction mixture is pre-denatured at 95°C for 5 min; followed by 25 cycles of 95°C for 15 s, annealing temperature for 30 s and 72°C for 30 s; and elongation at 72°C for 5 min.

Table C.2 Regular PCR primers for the *ail*, *foxA*, and *inv* genes

Primer	Sequence/ 5′ → 3′	Annealing temperature/°C	Amplification length/bp
ail-F	TAATGTGTACGCTGCGAG	57	351
ail-R	GACGTCTTACTTGCACTG		
foxA-F	GGTTCCTTGAGCGTATTGATG	58	1094
foxA-R	GGTCATCGGTTTCAGCAGTTT		
inv-F	CGGTACGGCTCAAGTTAATCTG	61	183
inv-R	CCGTTCTCCAATGTACGTATCC		

Bands are observed by 1.5% agarose gel electrophoresis after regular PCR.

Sequence determination of target gene: PCR products for the *ail*, *foxA*, and *inv* genes are sent to a commercial sequencing company for sequence determination of both directions.

C.3.2 Results interpretation

Interpretation of the nucleic acid test of the sample is shown in Table C.3. Both *foxA* and *ail* positive results indicate that the sample contains pathogenic *Y. enterocolitica*; only *foxA* positive results indicate that the sample contains nonpathogenic *Y. enterocolitica*; *inv* positive results indicate that the sample contains *Y. pseudotuberculosis*.

Table C.3 Results interpretation of nucleic acid test of sample

	*fox*A	*ail*	*inv*
pathogenic *Y. enterocolitica*	+	+	-
nonpathogenic *Y. enterocolitica*	+	-	-
Y. pseudotuberculosis	-	-	+

C.4 Bacterial isolation and culture from a sample

According to the different purposes of pathogen detection, a direct isolation or long-term cold enrichment strategy is adopted. For clinical samples of acute stage or epidemic samples, direct strain isolation from selective agar saves time; for daily surveillance or retrospective screening, a long-term cold enrichment strategy can be adopted to increase the isolation rate.

The same isolation and culture process is adopted for *Y. enterocolitica* and *Y. pseudotuberculosis*, which are distinguished by colony morphology and biochemical reaction of the strains.

C.4.1 Procedure of bacterial isolation and culture from a sample

Stool, rectal swab and whole blood samples are directly inoculated on plates or in a selective enrichment solution; tissues and blood clots are aseptically ground and inoculated on plates or in a selective enrichment solution. Strain isolation and culture refers to the following procedure (Fig. C.1).

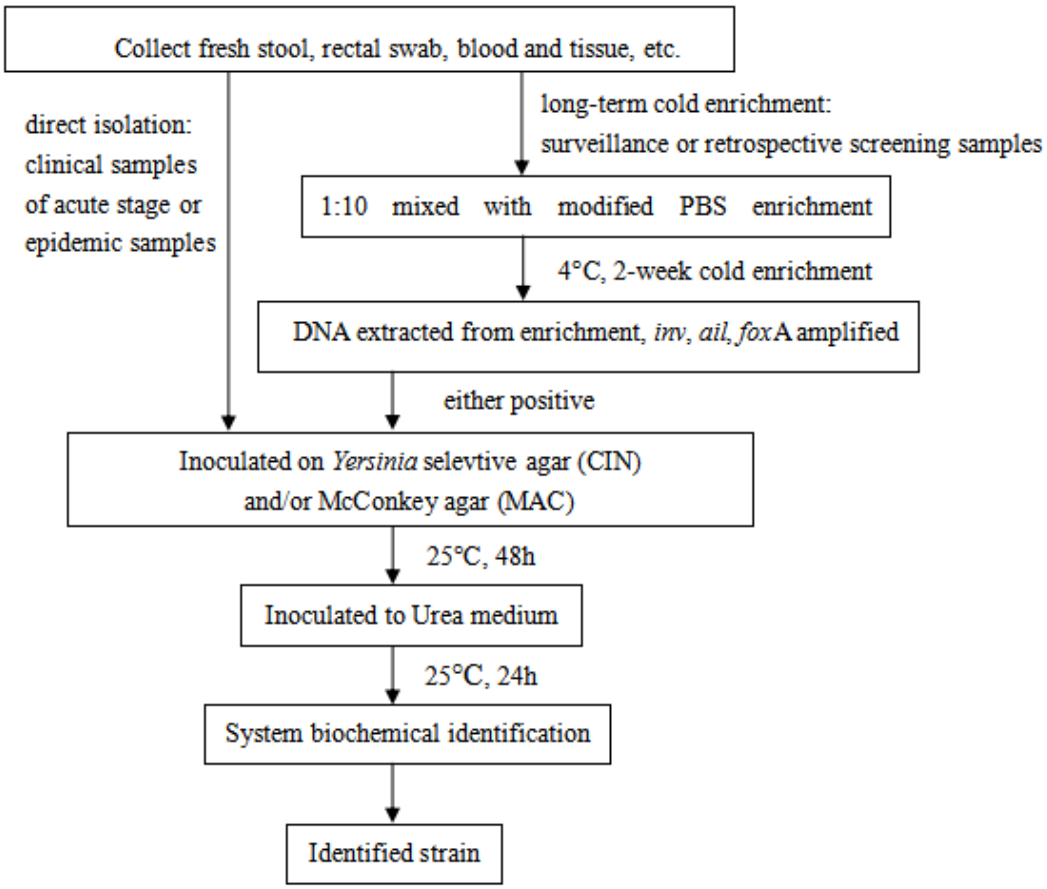

Figure C.1 Procedure of isolation and culture of *Y. enterocolitica* and *Y. pseudotuberculosis*

C.4.2 Sample cold enrichment

If a direct isolation strategy is selected, skip to step C.4.4.

If a long-term cold enrichment strategy is selected, the sample is inoculated in commercial Peptone Sorbitol Bile Broth (PSB) enrichment solution or commercial-modified Phosphate Saline

(modified PBS) at a ratio of no less than 1:10. The total volume of the enrichment solution is at least 6 mL, and the sample is shaken well. The culture is immediately placed at 4°C for 2 weeks of cold enrichment.

C.4.3 Molecular screening of enrichment solution

After 2 weeks of enrichment, the DNA of the enrichment solution is extracted and molecularly screened for *Yersinia* according to the method described in C.3. Samples positive for *foxA*, *ail*, or *inv* are inoculated on selective agar for further isolation. Samples negative for all three genes are regarded as free of *Y. enterocolitica* and *Y. pseudotuberculosis* and are normally not further submitted to strain isolation.

C.4.4 Selective agar inoculation

Samples that are selected for direct isolation or samples that are positive for PCR screening are inoculated on *Yersinia*-selective agar (CIN) or MacConkey agar (MAC) and streaked. The plates are cultured at 25°C immediately after inoculation.

Y. enterocolitica and *Y. pseudotuberculosis* can grow on a variety of intestinal selective media. They can grow under aerobic or anaerobic conditions. The growth temperature range of both species is wide, from 0°C to 45°C, and the optimal growth temperature is 25°C to 28°C. Ordinary LB agar or Brain Heart Infusion agar is used for general culture. *Y. enterocolitica* takes 18 h to 24 h, and *Y. pseudotuberculosis* takes 24 h to 48 h; the latter grows slower. The colonies of the two *Yersinia* species are similar, but those of *Y. pseudotuberculosis* are smaller. After culture at 25°C for 24 h, colonies of both species are small, moist and milky-white. The colony on the Brain Heart Infusion agar is darker in color. Both species grow evenly in broth, and no bacterial pellicle is formed normally.

It is recommended that the first inoculation of the sample proceeds for 48 h. On CIN, *Y.*

enterocolitica forms small and moist colonies with a diameter of approximately 1 mm to 2 mm that are deep rose-red and have bulges at the center with a narrow and translucent ring around it, which is called a "bull's eye" colony. A colony of *Y. pseudotuberculosis* on CIN is similar to that of *Y. enterocolitica*, but the colonies are smaller, the rose-red center is darker and the translucent ring is narrower.

After culture at 25°C for 24 h to 48 h on MacConkey agar, *Yersinia* forms small colonies with diameters of approximately 0.5 mm to 1 mm. The colonies are round, smooth, moist and translucent and are slightly pink in the center.

C.4.5 Biochemical screening - Urea utilization test

At least 5 suspected colonies are picked from each selective agar for biochemical identification, which are inoculated in urea liquid medium, vortexed thoroughly and cultured at 25°C. Urea medium changes to red when urea is utilized and can be observed successively at 2 h after inoculation. The end point of observation is 24 h.

C.4.6 System biochemical identification

A suspected strain that utilizes urea is re-cultured on Brain Heart Infusion agar or LB agar at 25°C to 28°C for biochemical identification. Manual biochemical identification strips, biochemical identification systems and mass spectrometers can be adopted to ultimately identify the genus and species of the strain.

It should be noted that the optimal culture temperature of *Yersinia* is 25°C to 28°C, whereas the culture temperature of the biochemical identification system is 37°C, which may change the results of some biochemical reactions and lead to identification error or unidentified results. *Y. enterocolitica* and *Y. pseudotuberculosis* may be missed or misdetected by mass spectrometers due to current database limitations.

At present, the BioMérieux manual biochemical identification strip (API20E) is the gold standard for the biochemical identification of *Yersinia*. In addition, there may be some cases of abnormal biochemical reactions, such as ornithine decarboxylase, citric acid or other reactions. When the colony is similar and other biochemical reactions are consistent with *Y. enterocolitica* or *Y. pseudotuberculosis*, manual single-tube biochemical reactions can be used for re-identification to avoid missing target strains.

The main differences in biochemical reactions between *Y. enterocolitica* and *Y. pseudotuberculosis* are shown in Table C.4.

Table C.4 Biochemical reactions comparison between *Y. enterocolitica* and *Y. pseudotuberculosis*

Biochemical reactions	*Y. enterocolitica*	*Y. pseudotuberculosis*	Biochemical reactions	*Y. enterocolitica*	*Y. pseudotuberculosis*
Motility 25°C	+	+	D-Glucose, gas	-	-
Motility 37°C	-	-	D-Mannitol	+	+
Urea	+	+	Sorbitol	+	-
H_2S	-	-	Sucrose	+	-
Oxidase	-	-	Raffinose	-	v
Indole	v	-	Melibiose	-	v
Methyl red	+	+	L-Rhamnose	-	+
Voges-Proskauer 25°C/37°C	v/-	-	Cellobiose	+	-
Citric acid	-	v	Inositol	v	-
Phenylalanine deaminase	-	-	Lactose	v	-
Ornithine decarboxylase	+	-	L-Arabinose	+	+
Lysine decarboxylase	-	-	Salicylic acid	v	v
Arginine decarboxylase	-	-	Esculin	v	+
Note: +: positive, -: negative, v: varied with strains.					

C.5 The storage and transportation of the bacterium

Y. enterocolitica and *Y. pseudotuberculosis* can survive for more than 2 weeks on plates or semi-solid media. They can be stored for at least 3 months to half a year on 0.5% Brain Heart

Infusion semi-solid medium, which can be stored or transported for short periods of time. Long-term preservation should be performed in 20% to 30% glycerin broth and frozen at -80°C, which can then be stored for more than ten years. A large amount of bacteria is beneficial for storing strains. Using porcelain beads for short-term or long-term preservation is not good for the recovery of strain.

C.6 Pathogen characteristics identification

The pathogen characteristics identification of *Y. enterocolitica* and *Y. pseudotuberculosis* can be performed according to the following method.

C.6.1 Serotyping

C.6.1.1 *Y. enterocolitica*

Y. enterocolitica can be classified into at least 70 serotypes according to the O antigen. However, only several of the common pathogenic serotypes can be identified by the current global commercial typing antisera (or monoclonal antibodies), including the O:1,2, O:3, O:5, O:8 and O:9 serotypes. The serotypes can be detected by slide agglutination with specific monoclonal antibodies and antisera and using saline as a control. The rough strains show self-coagulation in normal saline, which cannot be classified by serotype.

One strain of *Y. enterocolitica* may have multiple O antigens, such as O:5,27, O:1,2a,3, etc. As testing with only a few antisera may lead to the lack of detection of other antigens (factors), an agglutination test must be performed with all types of antisera to determine the serotype. There are common O antigens and cross-reactions, such as between O:9 *Y. enterocolitica* and *Brucella*.

The typical positive results of slide agglutination show some large agglomerated particles, and the liquid is completely clear. Sometimes, the positive agglutination result is not typical enough, as the liquid is still turbid with some small particles. The positive agglutination test results of a monoclonal antibody are different from those of an antisera, which show fine sand agglutination.

As the serum of a rabbit is a polyclonal antibody, nonspecific agglutination will exist. Quality evaluation is needed before a batch of diagnostic serum is used.

C.6.1.2 *Y. pseudotuberculosis*

At present, a total of 15 serotypes and 6 subtypes have been identified: O:1a, O:1b, O:1c, O:2a, O:2b, O:2c, O:3, O:4a, O:4b, O:5a, O:5b, O:6, O:7, O:8, O:9, O:10, O:11, O:12, O:13, O:14 and O:15. Pathogenic strains exist among each of the serotypes of *Y. pseudotuberculosis*, and both pathogenic and nonpathogenic strains can be found in the same serotype. Most of the O:1 to O:5 serotypes worldwide are pathogenic strains.

Serotypes of strains can be identified with PCR methods to avoid the limitations of typing serum.

Each target listed in Table C.5 is amplified with PCR to identify the serotype.

Table C.5 The serotype identification for *Y. pseudotuberculosis* with PCR

Serotype	Target gene										
	gmd-fcl	*ddhC-prt*	*manB*	*abe*	*wbyL*	*wbyH*	*ddhAB*	*wbyK*	*wzx*	*wzz-gsk*	*hemD-ddhD*
O:1a		+				+	+		+		
O:1b	+	+	+		+	+	+	+		+	+
O:1c	+		+			+	+	+	+		
O:2a				+			+				
O:2b	+		+	+			+				
O:2c			+	+			+				
O:3	+	+	+				+				
O:4a			+				+				
O:4b		+					+				
O:5a	+		+				+		+		
O:5b	+		+						+		
O:6							+				
O:7										+	
O:8		+	+				+				
O:9											
O:10										+	+
O:11	+		+					+	+		
O:12	+		+		+		+				
O:13	+		+		+		+				
O:14	+		+		+	+		+	+		

O:15	+	+	+			+	+		+		

The primer sequences are shown in Table C.6.

Table C.6 The primer sequences for *Y. pseudotuberculosis* serotyping

Target gene	Primer	Sequences/ 5′ → 3′	Amplification length/bp	Annealing temperature/°C
gmd-fcl	Ypf-14159	TCAAGATCGCCATGAGAC	1370	53
	Ypr-15549	AGGTTCATTCGTTGGTTC		
ddhC-prt	Ypf-5270	CGCATAGAAGAGTTTGTTG	1072	
	Ypr-6342	CTTTCGCCTGAAATTAGAC		
manB	Ypf-18740	GCGAGCCATAACCCAATAGAC	963	
	Ypr-19703	GCCACCCATCAAATTCCATAC		
abe	Abe1	AGAATAGTTCTGACTGGAGGAAG	775	
	Abe2	TCAGGAGCCATTACCTCATC		
wbyL	Ypf-17770	TTGGAGAAACAAACCTATCTGG	644	
	Ypr-18414	TTTGCATAAAAACGACATAGGC		
wbyH	Ypf-7170	CGTTATCCCAAAAAAGAGG	528	
	Ypr-7698	ATGGGAGACGCTTGTGATG		
ddhA-B	Ypf-3057	TGTCGCCTAAAGTTATCG	407	
	Ypr-3464	CGAATATCACCGATTTCC		
wbyK	Ypf-13231	CCGATTACCAGATTTTGAC	307	
	Ypr-13538	CAAAATTCTTATAACCACCACG		
wzx	Ypf-8576	GAAATTCGCATGTAAAAGCTATTG	105	
	Ypr-8681	GAACCTAGACTTACCACCCCCAAC		
wzz-gsk	Ypf-20511	GAAAAATACAGCGAGCAG	742	55
	Yerfb2	GAYTTGCGYTTACCAGGAAATTTCATTG		
hemD-ddhD	Ypf-913	CAATCCAATGAAGAGTCAG	181	
	Ypr-1094	CCCTATGACATAAAAACCC		

The amplification system and amplification procedure are shown in Table C.3.1.2.

C.6.2 Biotyping

C.6.2.1 *Y. enterocolitica*

Biochemical reactions are not only the main basis for identification of *Y. enterocolitica* but also the basis for biotyping. According to the biochemical reactions in Table C.7, the biotype of *Y. enterocolitica* can be divided into biotype 1A, 1B, 2, 3, 4 and 5 after incubation for 48 h at 25°C. The biotype of *Y. enterocolitica* is clinically significant. Most strains of biotype 1A are nonpathogenic, while most strains of biotype 1B, 2, 3, 4 and 5 are pathogenic.

Table C.7 Biotype index of *Y. enterocolitica*

Biochemical Reaction	Biotype					
	1A	1B	2	3	4	5
Lipase activity	+	+	-	-	-	-
Esculin hydrolysis	+	-	-	-	-	-
Salicin	+	-	-	-	-	-
Indole	+	+	(+)	-	-	-
Xylose	+	+	+	+	-	d
Trehalose	+	+	+	+	+	-
Nitrate Reduction	+	+	+	+	+	-
DNase	-	-	-	-	+	+
Prolidase	d	-	-	-	-	-
β-D-Glucosidase	+	-	-	-	-	-
Pyrazinamidase	+	-	-	-	-	-
Note: +: ≧90% strains are positive; d: 11% - 98% strains are positive; -: ≧90% strains are negative; (+): weakly positive.						

C.6.2.2 *Y. pseudotuberculosis*

Y. pseudotuberculosis can be divided into 4 biotypes by the tests for raffinose, melibiose and tannic acid utilization test (Table C.8).

Table C.8 Biotype index of *Y. pseudotuberculosis*

Biochemical Reaction	Biotype			
	1	2	3	4
Raffinose	-	-	-	+
Melibiose	+	-	-	+
Tannic acid	-	-	+	-

C.6.3 Virulence gene identification

C.6.3.1 *Y. enterocolitica*

The following 5 genes were tested by common PCR, and positive and negative controls were designed for all reactions:

ail (attachment invasion locus gene), *ystA* (heat-stable enterotoxin A gene of *Y. enterocolitica*) and *ystB* (heat-stable enterotoxin B gene of *Y. enterocolitica*), which are located on the chromosome; and *yadA* (adhesive gene) and *virF* (the transcriptional activator of the *Yersinia* yop regulon), which are located on a virulence plasmid (pYV).

The amplification system and program are shown in Table C.3.1.2. The amplification primers are shown in Table C.9.

Table C.9 PCR amplification primer sequences for the virulence genes of *Y. enterocolitica*

Primer	Sequence/5' → 3'	Amplification length/bp	Annealing temperature/°C
ail-F	TAATGTGTACGCTGCGAG	351	57
ail-R	GACGTCTTACTTGCACTG		
ystA-F	ATCGACACCAATAACCGCTGAG	79	61
ystA-R	CCAATCACTACTGACTTCGGCT		
ystB-F	GTACATTAGGCCAAGAGACG	146	61
ystB-R	GCAACATACCTCACAACACC		
yadA-F	CTTCAGATACTGGTGTCGCTGT	849	60
yadA-R	ATGCCTGACTAGAGCGATATCC	759[a]	
virF-F	GGCAGAACAGCAGTCAGACATA	561	63
virF-R	GGTGAGCATAGAGAATACGTCG		
[a] Amplification length of 1B/O:8 strains.			

Traditionally, *Y. enterocolitica* can be classified into pathogenic and nonpathogenic strains according to the virulence genes carried by chromosome and virulence plasmids. *ail* and *ystA,* which are on the chromosome, are necessary for pathogenic strains. Under natural conditions, pathogenic strains carry virulence plasmids, while under artificial passaging, virulence plasmids may be lost. Traditional nonpathogenic strains do not carry the above virulence genes, but some nonpathogenic strains carry *ystB* on their chromosome. The traditional pathogenic strain is the main pathogen of *Yersinia*. However, sporadic cases and even small outbreaks of *Yersinia* caused

by traditional nonpathogenic strains have been increasingly reported recently around the world.

Result interpretation (Table C.10):

Pathogenic *Y. enterocolitica*: *ail*+, *ystA*+, *ystB*-, *yadA*+, *virF*+ (possessing the virulence plasmid of *Yersinia*); *ail*+, *ystA*+, *ystB* -, *yadA*-, *virF*- (lacking the virulence plasmid of *Yersinia*).

Nonpathogenic *Y. enterocolitica*: *ail*-, *ystA*-, *ystB*+, *yadA*-, *virF*-; *ail*-, *ystA*-, *ystB*-, *yadA*-, *virF*-.

Table C.10 Pathogenicity determination of *Y. enterocolitica* strains

Strain pathogenicity	Chromosome			Virulence plasmid	
	ail	*ystA*	*ystB*	*yadA*	*virF*
Pathogenic strain	+	+	-	+	+
	+	+	-	-	-
Nonpathogenic strains	-	-	+	-	-
	-	-	-	-	-

C.6.3.2 *Y. pseudotuberculosis*

The following genes were tested by common PCR, and negative controls were established for all reactions: *inv, yadA, virF and ypmA, ypmB, ypmC* (encoding mitogens A, B, C from *Y. pseudotuberculosis*).

The amplification system and program are shown in Table C.3.1.2. The amplification primers are shown in Table C.11.

Table C.11 PCR amplification primer sequences for the virulence genes of *Y. pseudotuberculosis*

Primer	Sequence/5’ → 3’	Amplification length/bp	Annealing temperature/°C
inv-F	CGGTACGGCTCAAGTTAATCTG	183	61
inv-R	CCGTTCTCCAATGTACGTATCC		
yadA-F	CTTCAGATACTGGTGTCGCTGT	849	60
yadA-R	ATGCCTGACTAGAGCGATATCC		
virF-F	TCATGGCAGAACAGCAGTCAG	590	53
virF-R	ACTCATCTTACCATTAAGAAG		

ypmA-F	CACTTTTCTCTGGAGTAGCG	350	51
ypmA-R	GATGTTTCAGAGCTATTGTT		
ypmB-F	TTTCTGTCATTACTGACATTA	453	52
ypmB-R	CCTCTTTCCATCCATCTCTTA		
ypmC-F	ACACTTTTCTCTGGAGTAGCG	418	53
ypmC-R	ACAGGACATTTCGTCA		

C.6.4 Molecular typing—PFGE

Pulse-field gel electrophoresis (PFGE) is currently considered the gold standard for the molecular typing of *Y. enterocolitica*. In addition, multi-locus sequence typing (MLST) analysis and single-nucleotide polymorphisms (SNPs) analysis can be used as molecular typing methods.

The PFGE procedure for *Y. enterocolitica* and *Y. pseudotuberculosis* is the same as that for general intestinal bacteria. *Y. enterocolitica* is digested with NotI, and PFGE is performed with an electrophoresis pulse time of 2 s ~ 20 s and electrophoresis time of 18 h ~ 19 h (10-lane gel: 18 h, 15-lane gel: 19 h). *Y. pseudotuberculosis* is digested with NotⅠ or FseⅠ, and PFGE is performed with an electrophoresis pulse time of NotⅠ: 2 s ~ 18 s, FseⅠ: 2 s～35 s and electrophoresis time of 18 h ~ 19 h (10-lane gel: 18 h, 15-lane gel: 19 h).

C.7 Biosafety requirements

According to the former Ministry of Health "List of Pathogenic Microorganisms Infecting Human Beings" ([2006] No. 15 published by Ministry of Health), the degree of damage caused by *Y. enterocolitica* and *Y. pseudotuberculosis* belongs to the third category. The operations on samples and pure cultures, such as sample testing, bacterial isolation and culture, biochemical identification, serotyping, nucleic acid extraction, and so on, are conducted in a BSL-2 laboratory and shipped in a Class B (UN3373) package.

Note: After the new version of the "List of Pathogenic Microorganisms Infecting Human Beings" is released, these procedures should be implemented in accordance with the new version.

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