U.S. FOOD & DRUG ADMINISTRATION

Bacteriological Analytical Manual

Chapter 8: *Yersinia enterocolitica*

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Revision History

- October 2017: The instructions for "F. Identification" was modified to clarify the procedure.

Introduction

Yersinia enterocolitica and bacteria that resemble it are ubiquitous, being isolated frequently from soil (2, 18), water (2, 10, 17), animals (2, 18), and a variety of foods (5, 6, 15). They comprise a biochemically heterogeneous group that can grow at refrigeration temperatures (a strong argument for use of cold enrichment). Based on their biochemical heterogeneity and DNA relatedness, members of this group were separated into four species: *Y. enterocolitica*, *Y. intermedia*, *Y. frederiksenii*, and *Y. kristensenii* (9). Through additional revisions, the genus *Yersinia* has grown to include eleven species (2, 9, 10, 51), three of which are potentially pathogenic to humans: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. Of these, *Y. enterocolitica* is most important as a cause of foodborne illness.

Y. enterocolitica strains and related species can be separated serologically into groups based on their heat-stable somatic antigens. Wauters (51) described 54 serogroups for *Y. enterocolitica* and related species. Aleksic and Bockemuhl (1) proposed simplifying this to 18 serogroups within the *Y. enterocolitica* species. Presently, pathogenic strains belonging to serogroups O:1, 2a, 3; O:2a,3; O:3; O:8; O:9; O:4,32; O:5,27; O:12,25; O:13a,13b; O:19; O:20; and O:21 have been identified. Therefore, pathogenic strains can belong to diverse serogroups. Serogroups that predominate in human illness are O:3, O:8, O:9, and O:5,27.

The association of human illness with consumption of *Y. enterocolitica*-contaminated food, animal wastes, and unchlorinated water is well documented (5, 6,17). This organism may survive and grow during refrigerated storage. Because contamination is possible at the manufacturing site (5) or in the home, refrigerated foods are potential vehicles (6).

The mechanisms of pathogenicity in the enteropathogenic *Yersinia* are complex and have served as research models for understanding the infectious process in many enteropathogenic bacteria. They include a number of both chromosomally and plasmid determined factors. Chromosomal determinates include: invasion of host cells (*ail* for *Y. enterocolitica* and *inv* for *Y. pseudotuberculosis*), iron complexing and uptake proteins *(irps)*, and heat-stable enterotoxin (ystA). Factors carried on the 70 kb virulence plasmid (pYV for *Y. enterocolitica* and pIB1 for *Y. pseudotuberculosis*) include: the *Yersinia* outer proteins *(yops)*, low calcium response (lcr), *Yersinia* adherence protein *(yadA)*, and the temperature dependent transcriptional regulator of many of the other plasmid genes *(virF)* (38).

A number of virulence tests have been proposed to distinguish potentially pathogenic *Y. enterocolitica*. The heat-stable enterotoxin (ST) produced *in vitro* by some strains of *Y. enterocolitica* and related species can be detected by intragastric injection of cultural filtrates in suckling mice and is very similar to *Escherichia coli* ST (12). However, *Yersinia* spp. produce ST *in vitro* only at temperatures below 30°C. Many environmental strains of *Yersinia* produce this protein, whereas some otherwise fully virulent strains of Y. enterocolitica do not. More recently, a specific subtype of the ST gene, *stA* has been more closely associated with pathogenic Y. enterocolitica strains (44). Still, the role of ST in the disease process of *Yersinia* remains uncertain.

Yersinia spp. that cause human yersiniosis carry a plasmid (~70kb) (19, 29, 56) that is associated with a number of traits related to virulence: autoagglutination in certain media at 35-37°C (8, 30); inhibition of growth in calcium-deficient media (19) and binding of crystal violet dye (11) at 35-37°C; increased resistance to normal human sera (39); production of a series of outer membrane proteins at 35-37°C (41); ability to produce conjunctivitis in guinea pig or mouse (Sereny test) (49, 56); and lethality in adult and suckling mice by intraperitoneal (i.p.) injection of live organisms (8, 14, 43, 45). The plasmid associated with virulence can be detected by gel electrophoresis or DNA colony hybridization (22). Recent evidence, however, indicates that presence of plasmid alone is not sufficient for the full expression of virulence in *Yersinia* (21,42,48). The intensity of some plasmid-mediated virulence properties such as mouse lethality and conjunctivitis is variable, depending on the genes carried on the bacterial chromosome (39-41, 46) and the serogroup, suggesting that chromosomal genes also contribute to *Yersinia* virulence.

Virulent strains of *Yersinia* also invade mammalian cells such as HeLa cells in tissue culture (29). However, strains that have lost other virulent properties retain HeLa invasiveness, because the invasive phenotype for mammalian cells is encoded by chromosomal loci. Two chromosomal genes of *Y. enterocolitica*, *inv* and *ail*, which encode the phenotype for mammalian cell invasion, have been identified (36,37). Transfer of these genetic loci into *E. coli* confers the invasive phenotype to the *E. coli* host (36). The *inv* gene allows high level *Yersinia* invasion of several tissue culture cell lines (36). However, Southern blot analyses show that *inv* gene sequences are present on both tissue culture invasive and noninvasive isolates (37, 46). Although this suggests that the *inv* gene in *Y. enterocolitica* may not be directly correlated with invasiveness, genetic evidence shows that *inv* genes are nonfunctional in the noninvasive isolates (40). The *ail* gene shows greater host specificity with regard to cell invasion and appears to be present only on pathogenic *Y. enterocolitica.* In disease-causing strains, all virulent *Y. enterocolitica* isolates were shown to be tissue culture-invasive and to carry the *ail* gene (36, 41). The *ail* locus, therefore, may be an essential chromosomal virulence factor in *Y. enterocolitica* (37, 46). DNA colony hybridization and PCR tests can be used to confirm the presence of these genes related to pathogenicity in isolated strains from food samples (16, 35, 38).

Y. pseudotuberculosis is less ubiquitous than *Y. enterocolitica*, and although frequently associated with animals, has only rarely been isolated from soil, water, and foods (18,50). Among *Y. pseudotuberculosis* strains there is little or no variation in biochemical reactions, except with the sugars melibiose, raffinose, and salicin. Serologically (based on a heat-stable somatic antigen), the *Y. pseudotuberculosis* strains are classified into six groups, each serogroup containing pathogenic strains. Gemski et al. (20) reported that serogroup III strains harbor a 70kb plasmid as do serogroup II strains that are lethal to adult mice. The association of

yersiniosis in humans with the presence of a 70kb plasmid in *Y. pseudotuberculosis* has been well established (38, 42).

Virulence genes present on the chromosome of *Y. pseudotuberculosis* have also been identified (24,25). The *inv* gene of *Y. pseudotuberculosis* is homologous with that of *Y. enterocolitica*, and encodes for an invasion factor for mammalian cells. Transfer of *inv* gene into *E. coli* K-12 resulted in the expression of the invasive phenotype in *E. coli* (25). The *inv* gene is thermoregulated (23,27); it encodes for a 103 Kdal protein, invasin, which binds to specific receptors on mammalian cells and facilitates the entry of *Y. pseudotuberculosis* into host tissue (26). Tests for *Y. pseudotuberculosis* virulence are not as abundant as those for *Y. enterocolitica*; however, tissue cell-invasive and plasmid-carrying isolates of *Y. pseudotuberculosis* may be identified by DNA colony hybridization or PCR (38).

A convenient *in vitro* test for potential virulence in *Y. enterocolitica* and *Y. pseudotuberculosis* strains is the formation of small highly convex colonies with the uptake of congo red dye when grown at 35-37°C but not at 26°C (11).

Numerous animal models have been used to assess virulence of *Yersinia* species for humans. These models include keratoconjunctivitis in Guinea pigs (Sereny test), enterocolitis to rabbits, lethality to gerbils, and to suckling and adult mice (38). Treatment of mice with iron and an iron chelator, desferrioximine B, prior to i.p. infection of adult mice increases the susceptibility of mice to infection by *Yersinia* (45).

A. Equipment and materials

1. Incubators, maintained at 10 ± 1°C, ± 35-37°C
2. Blender, Waring or equivalent, 8000 rpm, with 500 ml-1 liter jar
3. Sterile petri dishes, 15 × 100 mm
4. Microscope, light 900× and illuminator
5. Disposable borosilicate tubes, 10 × 75mm; 13 × 100mm.
6. Wire racks to acommodate 13×100mm tubes.
7. Vortex mixer.

B. Media

1. Peptone sorbitol bile broth (PSBB) (M120)
2. MacConkey agar (M91) (use mixed bile salts; BBL Mac agar and DIFCO Mac CS are acceptable)
3. Celfsulodin-irgasan-novobiocin (CIN) agar (M35)

4. Bromcresol purple broth (M26) supplemented individually with the following carbohydrates, each at 0.5%: mannitol, sorbitol, cellobiose, adonitol, inositol, sucrose, rhamnose, raffinose, melibiose, salicin, xylose, and trehalose
5. Christensen's urea agar (M40) (plated media or slants)
6. Phenylalanine deaminase agar (M123) (plated media or slants)
7. Motility test medium (M103). Add 5 ml of 1% 2,3,5-triphenyl tetrazolium chloride per liter before autoclaving.
8. Tryptone broth, 1% (M164)
9. MR-VP broth (M104)
10. Simmons citrate agar (M138)
11. Veal infusion broth (M173)
12. Bile esculin agar (M18)
13. Anaerobic egg yolk agar (M12)
14. API 20E or Vitek GNI
15. Trypticase (tryptic) soy agar with yeast extract (TSAYE) (M153)
16. Lysine arginine iron agar (LAIA) (M86)
17. Decarboxylase basal medium (Falkow) (M44) supplemented with 0.5% ornithine
18. Congo Red-brain heart infusion agarose (CRBHO) (M41)
19. Pyrazinamidase agar slants (M131)
20. PMP broth (M125)
21. β-D-glucosidase test (**see** instructions at end of chapter)

C. Reagents

1. Gram stain reagents (R32)
2. Voges-Proskauer (VP) test reagents (R89)
3. Ferric chloride, 10% in distilled water (R25)
4. Oxidase test reagent (R54)
5. Saline, 0.5% (sterile) (R66)

6. Kovacs' reagent (R38)
7. 0.5% Potassium hydroxide in 0.5% NaCl, freshly prepared
8. Mineral oil, heavy grade, sterile (R46)
9. API 20E system or Vitek system with GNI cards (bioMerieux)
10. 1% Ferrous ammonium sulfate

D. Enrichment

The following simplified procedure for isolating *Yersinia* from food, water, and environmental samples is recommended.

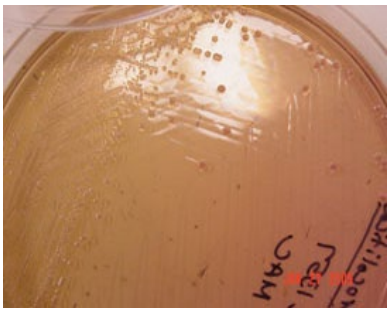
1. Analyze samples promptly after receipt, or refrigerate at 4°C. (Freezing of samples before analysis is not recommended, although *Yersinia* have been recovered from frozen products.) Aseptically weigh 25 g sample into 225 ml PSBB. Homogenize 30 s and incubate at 10°C for 10 days.
2. If high levels of *Yersinia* are suspected in product, spread-plate 0.1 ml on MacConkey agar (15,55) and 0.1 ml on CIN agar (47,54) before incubating broth. Also transfer 1 ml homogenate to 9 ml 0.5% KOH in 0.5% saline (4), mix for 2-3 seconds, and spread-plate 0.1 ml on MacConkey and CIN agars. Incubate agar plates at 30°C for 1-2 days.
3. On day 10, remove enrichment broth from incubator and mix well. Transfer one loop-full of enrichment to 0.1 ml 0.5% KOH in 0.5% saline and mix for 2-3 s (4). Successively streak one loopful to MacConkey plate and one loopful to CIN plate. Transfer additional 0.1 ml enrichment to 1 ml 0.5% saline and mix 5-10 s before streaking, as above. Incubate agar plates at 30°C for 1-2 days.

E. Isolation of *Yersinia*

Examine CIN plates after 1 day incubation. Select small (1-2 mm diameter) colonies having deep red center with sharp border surrounded by clear colorless zone with entire edge.

Y. enterocolitica on MacConkey agar

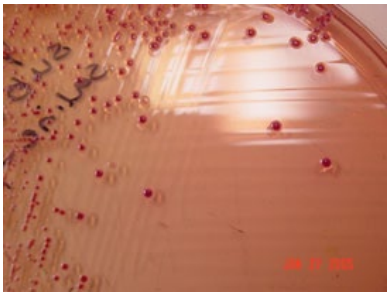
Examine MacConkey agar plates after 1 to 2 days incubation. Reject red or mucoid colonies. Select small (1-2 mm diameter) flat, colorless, or pale pink colonies.



- Lactose negative colonies
- flat, colorless, or pale pink
- 1-2 mm diameter

Y. enterocolitica on YSA (CIN) agar

Inoculate each selected colony into LAIA slant (53), Christensen's urea agar plate or slant, and bile esculin agar plate or slant by stabbing with inoculation needle. Incubate 48 h at RT. Isolates giving alkaline slant and acid butt, no gas and no H_2S (KA− −) reaction in LAIA, which are also urease-positive, are presumptive *Yersinia*. Discard cultures that produce H_2S and/or any gas in LAIA or are urease-negative. Give preference to typical isolates that fail to hydrolyze (blacken) esculin.

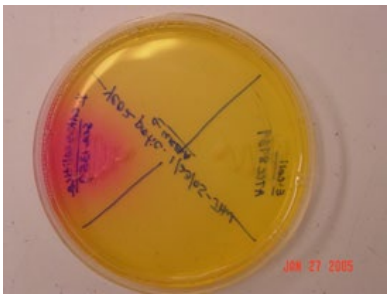


- deep red center
- Surrounded by clear, colorless zone
- 1-2 mm diameter

LAIA Slant

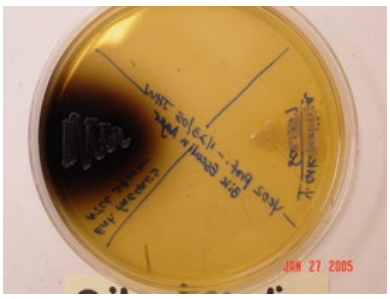
- *Y. enterocolitica* (left) = K A − −
- Salmonella (right) = K K + −

Christensen's Urea agar



- Y. ent. = pink color (urease postitive)
- E. coli = no color (urease negative)

Bile Esculin agar



- Y. ent. (except biotype 1A) are esculin negative (absence of black color)
- Ent. faecalis = esculin positive (black color)

F. Identification

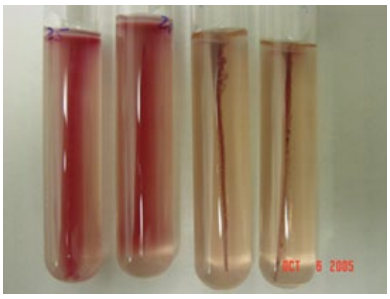
Using growth from LAIA slant, streak culture to one plate of TSAYE and incubate at RT. Use growth on TSAYE to check for culture purity and do oxidase test and Gram stain. From colonies on TSAYE, inoculate an agar media containing egg yolk such as Anaerobic egg yolk (AEY) agar for lipase reaction (at 2-5 days, incubated aerobically at RT). Also, inoculate the following biochemical test media and incubate all at RT for 3 days (except one motility test medium and one MR-VP broth, which are incubated at 35-37°C for 24 h).

1. Decarboxylase basal medium (Falkow) (M44), supplemented with each of 0.5% lysine, arginine, or ornithine; overlay with sterile mineral oil

2. Phenylalanine deaminase agar (M123)

3. Motility test medium (semisolid) (M103), 22-26°C and 35-37°C

Motility Test Medium with TTC



   - Y. ent. are motile at 25°C (2 left tubes) and non-motile at 35°C (2 right tubes)

4. Tryptone broth (M164)

5. Indole test (**see** instructions at end of chapter)

6. MR-VP broth (M104). RT for autoagglutination test (**see** H1, below), followed by V-P test (48 h) (**see** instructions at end of chapter); 35-37°C for autoagglutination test (**see** H-1)

7. Bromcresol purple broth (M26) with 0.5% of the following filter-sterilized carbohydrates: mannitol, sorbitol, cellobiose, adonitol, inositol, sucrose, rhamnose, raffinose, melibiose, salicin, trehalose, and xylose

8. Simmons citrate agar (M138)

9. Veal infusion broth (M173)

10. Use API 20E system or Vitek GNI for biochemical identification of Yersinia. Follow manufacturer's instructions. These systems are generally reliable to identify Yersinia to genus level but are generally unreliable in identification of Yersinia to species level (3, 32). Use conventional biochemical tests for speciation and biotyping of presumptive virulent isolates. Biochemical tests that are important for speciation within the genus

Yersinia are fermentation of sucrose, rhamnose, raffinose and melibiose and the utilization of citrate (Table 1). Biochemical tests important for biotyping are fermentation of salicin, xylose and trehalose along with VP reaction, lipase, esculinase, β-D-Glucosidase, and pyrazinamidase (Table 2).

11. Pyrazinamidase agar slants (48 h) (**see** instructions at end of chapter)

12. β-D-glucosidase test (30°C, 24 h) (**see** instructions at end of chapter)

13. Lipase test. When grown on agar media containing egg yolk such as Anaerobic egg yolk agar, colonies may exhibit lipase activity. A positive reaction is indicated by oily, iridescent, pearl-like colony surrounded by precipitation ring and outer clearing zone.

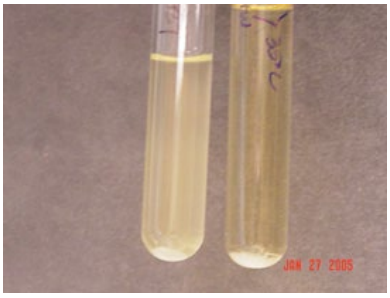
G. Interpretation

Yersinia are oxidase-negative, Gram-negative rods. Use Tables 1 and 2 to identify species and biotype of Yersinia isolates. Currently only strains of Y. enterocolitica biotypes 1B, 2, 3, 4, and 5 are known to be pathogenic. These biotypes and Y. enterocolitica biotype 6 and Y. kristensenii do not rapidly (within 24 h) hydrolyze esculin or ferment **salicin** (Tables 1 and 2). However, Y. enterocolitica biotype 6 and Y. kristensenii are relatively rare; they can be distinguished by failure to ferment sucrose, and they are pyrazinamidase-positive (28). Hold Y. enterocolitica isolates which are within biotypes 1B, 2, 3, 4, and 5 for further pathogenicity tests.

H. Pathogenicity testing

1. **Autoagglutination test**. The MR-VP tube incubated at RT for 24 h should show some turbidity from bacterial growth. The 35-37°C MR-VP should show agglutination (clumping) of bacteria along walls and/or bottom of tube with clear supernatant fluid. Isolates giving this result are presumptive positive for the virulence plasmid. Any other pattern for autoagglutination at these two temperatures is considered negative.

   MRVP Agglutination Test

   - When grown in MRVP broth at 25° C, pathogenic Y. ent. displays diffuse growth (left tube) but at 35°C cells agglutinate and settles to the bottom (right tube).

2. **Freezing cultures**. Plasmids that determine traits related to pathogenicity of Yersinia can be spontaneously lost during culture above 30°C or with lengthy culture and

passage below 30°C in the laboratory. It is important, therefore, to immediately freeze presumptive positive cultures to protect plasmid content. Inoculate into veal infusion broth and incubate 48 h at RT. Add 10% sterile glycerol (e.g., 0.3 ml in 3 ml veal infusion broth) and freeze immediately. Storage at -70°C is recommended.

3. **Low calcium response Congo Red agarose virulence test**. Inoculate test organism into BHI broth. Incubate overnight at 25-27°C. Make decimal dilutions in physiologic saline to obtain 1,000 cells/ml. Spread-plate 0.1 ml of appropriate dilution on each of two Congo Red agarose plates. Incubate one at 35°C and one at 25°C. Examine at 24 and 48 h. Presumptive plasmid-bearing Y. enterocolitica will appear as pinpoint, round, convex, red, opaque colonies. Plasmidless Y. enterocolitica will appear as large, irregular, flat, translucent colonies.

   Y. enterocolitica on CRBHO After 24h at 35° C

   - Plasmid bearing Y. ent. colonies are pinpoint convex, red, opaque.
   - Plasmidless colonies are large, irregular, flat, and translucent.

4. **Intraperitoneal infection of adult mice pretreated with iron dextran and desferrioxamine B.** A positive result from any of the in vitro pathogenicity tests (H, 1-3 above) is strong evidence of pathogenicity. These results may be confirmed by a biological test using the i.p. infection of adult mice which have been pretreated with iron dextran and the iron sequestering siderophore, desferrioxamine B. This test is described elsewhere in detail (13, 53) and is omitted here for brevity and because few labs have the facilities to perform bioassays.

5. **Invasiveness**. An in vitro HeLa cell assay is available for screening Yersinia isolates for invasive potential (33, 34). Acridine orange is used to stain infected HeLa cell monolayers, which are then examined under fluorescence microscope for the presence of intracellular Yersinia (33, 34). This in vitro staining technique can be used to determine invasiveness in both Y. enterocolitica and Y. pseudotuberculosis (16).

I. Interpretation

A positive reaction for any of the pathogenicity tests in H, 1-4 above can be taken as evidence of potential pathogenicity for a Y. enterocolitica or Y. pseudotuberculosis isolated strain.

J. Yersinia pseudotuberculosis

Generally, all Y. pseudotuberculosis strains are biochemically homogeneous except for production of acid from melibiose, raffinose, and salicin. Y. pseudotuberculosis heat-stable somatic antigens are also used to subgroup the species. At present there are six serogroups represented by Roman numerals I-VI. Serogroups I, II, III, and IV have subtypes, but antiserum to one serogroup type will cross-react with the subtype strain and vice versa. Strains belonging to serogroups II and III are lethal when fed to adult mice even though these strains do not elaborate lipase. HeLa cell-invasive strains are esculin-positive, which is contrary to findings with Y. enterocolitica. Y. pseudotuberculosis strains harbor a 41-48 Mdal plasmid and will autoagglutinate at 37°C. Association of yersiniosis in humans with the presence of a plasmid has been established (38).

1. **Enrichment**. Aseptically weigh 25 g sample into 225 ml PMP broth (17). Homogenize for 30 s and incubate at 4°C for 3 weeks. At 1, 2, and 3 weeks, mix enrichment well. Transfer 0.1 ml enrichment to 1 ml 0.5% KOH in 0.5% NaCl and mix for 5-10 s. Successively streak one loopful to MacConkey agar plate and one loopful to CIN agar plate. Streak one additional loopful directly from enrichment broth to one MacConkey and one CIN agar plate. Incubate agars at RT.

2. **Isolation and identification**. Continue as in E-H, above, noting biochemical differences (Table 1). Notably, Y. pseudotuberculosis strains are ornithine-, sorbitol-, and sucrose-negative.

Table 1. Biochemical Characteristics[a] of Yersinia species (2, 9 ,10 ,52)

Reaction	**Yersinia species** Y. pestis	**Yersinia species** Y. pseudo-tuberculosis	**Yersinia species** Y. enterocolitica	**Yersinia species** Y. intermedia	**Yersinia species** Y. frederiksenii	**Yersinia species** Y. kristensenii	**Yersinia species** Y. aldovae	**Yersinia species** Y. rohdei	**Yersinia species** Y. mollaretii	**Yersinia species** Y. bercovieri	**Yersinia species** Y. ruckeri
Lysine	-	-	-	-	-	-	-	-	-	-	
Arginine	-	-	-	-	-	-	-	-	-	-	
Ornithine	-	+[c]	+	+	+	+	+	+	+	+	
Motility at RT (22-26°C)	+	+	+	+	+	+	+	+	+	+	
35-37°C	-	-	-	-	-	-	-	-	-	-	
Urea	+	+	+	+	+	+	+	+	+	-	
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-	
Mannitol	+	+	+	+	+	+	+	+	+	+	
Sorbitol	-	+	+	+	+	+	+	+	+	-	
Cellobiose	-	+	+	+	+	-	+	+	+	-	
Adonitol	-	-	-	-	-	-	-	-	-	-	
Inositol	-	+/-(+)	+/-(+)	+/-(+)	+/-(+)	+	-	+/-	-		
Sucrose	-	+[c]	+	+	-	-	+	+	+	-	
Rhamnose	+	-	+	+	-	+	-	-	-	-	
Raffinose	+/-	-	+	-	-	-	+/-	-	-	-	
Melibiose	+/-	-	+	-	-	-	+/-	-	-	-	
Simmons citrate	-	-	+/-	+/-	-	-	+	-	-	+	
Voges- Proskauer	-	+/-(+)	+	+	-	+	-	-	-	-	
Indole	-	+/-	+	+	+/-	-	-	-	-	-	
Salicin	+/-	+/-	+	+	-(+/-)	-	-	+/-	(+)		
Esculin	+	+/-	+	+	-	+	-	(+)	(+)/-	-	
Lipase	-	+/-	+/-	+/-	+/-	+/-	-	-	-		
Pyrazinamidase	-	+/-	+	+	+	+	+	+	+		

[a] + = positive after 3 days at RT, (+) = positive after 7 days at RT.
[b] Some strains of Y. intermedia are negative for either Simmons citrate, rhamnose, and melibiose, or raffinose and Simmons citrate.
[c] Some biotype 5 strains are negative.

Table 2. Biotype scheme[a] for Y. enterocolitica

Biochemical test	Reaction for biotypes[b] **1A**	Reaction for biotypes[b] **1B**	Reaction for biotypes[b] **2**	Reaction for biotypes[b] **3**	Reaction for biotypes[b] **4**	Reaction for biotypes[b] **5**	Reaction for biotypes[b] **6**
Lipase	+	+	-	-	-	-	-
Esculin/salicin (24 h)	+/-	-	-	-	-	-	-
Indole	+	+	(+)	-	-	-	-
Xylose	+	+	+	+	-	V	+
Trehalose	+	+	+	+	+	-	+
Pyrazinamidase	+	-	-	-	-	-	+
β -D-Glucosidase	+	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+/-[c]	+	(+)	-

[a] Based on Wauters (51).
[b] ( ) = Delayed reaction; V = variable reactions.
[c] Biotype of serotype O:3 found in Japan.

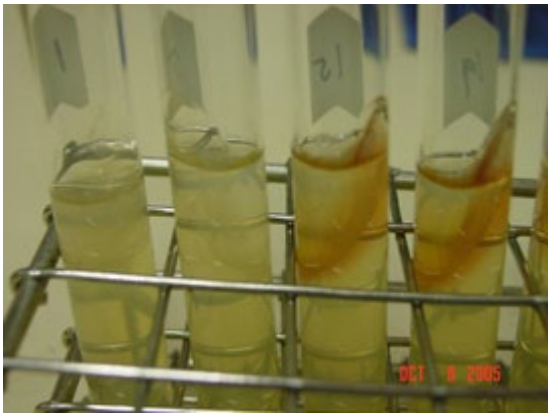
Instructions for Yersinia Identification Tests

Phenylalanine deaminase agar test: Add 2-3 drops 10% ferric chloride solution to growth on agar slant. Development of green color is positive test.

Indole test: Add 0.2-0.3 ml Kovacs' reagent. Development of deep red color on surface of broth is positive test.

V-P test: Add 0.6 ml **alpha**-naphthol and shake well. Add 0.2 ml 40% KOH solution with creatine and shake. Read results after 4 h. Development of pink-to-ruby red color in medium is positive test.

Pyrazinamidase test: After growth of culture on slanted pyrazinamidase agar at RT, flood 1 ml of 1% freshly prepared ferrous ammonium sulfate over slant. Development of pink color within 15 min is positive test, indicating presence of pyrazinoic acid formed by pyrazinamidase enzyme.



- Flood 1ml of 1% freshly prepared ferrous ammonium sulfate over slant. Pink color within 15 min is +

- (right 2 test tubes= positive, left 2 tubes = negative).

Beta-D-Glucosidase test: Add 0.1 g 4-nitrophenyl-**beta**-D-glucopyranoside to 100 ml 0.666 M NaH_2PO_4 (pH 6). Dissolve; filter-sterilize. Emulsify culture in physiologic saline to McFarland Turbidity Standard No. 3. Add 0.75 ml of culture to 0.25 ml of test medium. Incubate at 30°C overnight. A distinct yellow color indicates a positive reaction.

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