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Pyrazinamidase, CR-MOX Agar, Salicin Fermentation-Esculin Hydrolysis, and D-Xylose Fermentation for Identifying Pathogenic Serotypes of *Yersinia enterocolitica*

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We evaluated several simple laboratory tests that have been used to identify pathogenic serotypes of *Yersinia enterocolitica* or to indicate the pathogenic potential of individual strains. A total of 100 strains of *Y. enterocolitica* were studied, including 25 isolated during five outbreak investigations, 63 from sporadic cases, and 12 from stock cultures. The pyrazinamidase test, which does not depend on the *Yersinia* virulence plasmid, correctly identified 60 of 63 (95% sensitivity) strains of pathogenic serotypes and 34 of 37 (92% specificity) strains of nonpathogenic serotypes. Salicin fermentation-esculin hydrolysis (25°C, 48 h) correctly identified all 63 (100% sensitivity) strains of the pathogenic serotypes and 34 of 37 (92% specificity) strains of the nonpathogenic serotypes. The results of the pyrazinamidase and salicin-esculin tests disagreed for only 7 of the 100 strains of *Y. enterocolitica*, and these would require additional testing. Congo red-magnesium oxalate (CR-MOX) agar determines Congo red dye uptake and calcium-dependent growth at 36°C, and small red colonies are present only if the strain contains the *Yersinia* virulence plasmid. This test has proven to be extremely useful for freshly isolated cultures, but only 15 of 62 strains of pathogenic serotypes that had been stored for 1 to 10 years were CR-MOX positive. None of the 16 strains of *Y. enterocolitica* serotype O3 fermented D-xylose, so this test easily differentiated strains of this serotype, which now appears to be the most common in the United States. Although antisera that can actually be used to serotype strains of *Y. enterocolitica* are not readily available, the four simple tests described above can be used to screen for pathogenic serotypes.

Since it was first described in 1939, *Yersinia enterocolitica* has become well established as an enteric pathogen (4–7). However, there is considerable confusion in the literature because not all strains of this species can cause intestinal infections. Unlike *Salmonella* and *Shigella* species, which are intrinsic pathogens (essentially all strains can cause enteric infections), there is strain-to-strain variation in the pathogenicity of *Y. enterocolitica* (4, 6, 7, 12, 14, 19). A number of studies have shown an excellent correlation between the serotype and biotype of *Y. enterocolitica* and its ability to cause infections of the intestinal tract and to invade tissue (4, 6, 7, 12, 14, 21).

Many different tests and methods have been used over the years to define the pathogenic potential of *Y. enterocolitica* strains, but it has only recently been realized that an important component of virulence in this species is determined by a plasmid that is easily lost (10, 11, 19, 21). For this reason, there is confusion in the literature on how well the various methods identify pathogenic serotypes and determine pathogenic potential.

In 1985, Kandolo and Wauters (10) described the pyrazinamidase test as a way of differentiating pathogenic from nonpathogenic serotypes of *Y. enterocolitica*. In 1989, Riley and Toma (16) described Congo red-magnesium oxalate (CR-MOX) agar, a medium that allows easy recognition of *Y. enterocolitica* colonies that contain the *Yersinia* virulence plasmid. This medium determines two properties that are plasmid dependent, Congo red dye uptake and calcium-dependent growth at 36°C (see Fig. 1). In this report, we describe our experience with these two simple tests using strains isolated in the United States and show their correlation with serotype, salicin fermentation-esculin hydrolysis (salicin-esculin), and some other simple laboratory tests used to evaluate pathogenic potential (Table 1).

MATERIALS AND METHODS

Nomenclature. In this report, we use the term "pathogenic serotype" to refer to 11 O-antigen groups of the *Y. enterocolitica* serotyping schema. Strains belonging to these serotypes are generally recognized as being enteric pathogens on the basis of their occurrence in outbreaks and sporadic cases, pathogenicity in animal models, or the ability to invade tissue or animal cells in tissue culture (4, 6, 7, 14). Strains of serotypes O3, O9, and O5,27 are well documented as enteric pathogens in humans and are the most important serotypes found in Europe and much of the rest of the world (4, 6, 7). Until recently, these three serotypes were rare in the United States (2, 5, 13), where the pathogenic serotypes O4,32; O8; O13a,13b; O18; O20; and O21 are usually found (2–6, 12, 13). These six serotypes have been designated the pathogenic American serotypes (7, 10). Strains of two additional serotypes cause disease in animals: serotype O1,2,3 causes disease in chinchillas and serotype O2,3 causes disease in hares (4, 7). Serotype O1,2,3 has also been isolated from human clinical infections (16).

Strains and stock cultures. The strains of *Yersinia* spp. studied are described in more detail in Tables 2 through 4. Stock cultures were prepared at the time the culture was

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TABLE 1. Properties of *Y. enterocolitica* strains[a]

Property	Pathogenic serotypes with virulence plasmid:		Nonpathogenic serotypes
	Present	Absent[b]	
Small red colonies on CR-MOX agar	+	−	−
Temp-dependent autoagglutination	+	−	−
Invasiveness (cell culture)	+	+	−
Pyrazinamidase production	−	−	+
Salicin fermentation	−	−	+
Esculin hydrolysis	−	−	+
Belong to serotypes O1,2,3; O2,3; O3; O4,32; O5,27; O8; O9; O13a,13b; O18; O20; or O21	+	+	−
Belong to other serotypes	−	−	+

[a] Based on published reports (10, 14–16, 20).
[b] Presumably, the virulence plasmid was originally present, but it was lost during subculture or storage.

received (1978 to 1983 for outbreaks, 1982 to 1987 for sporadic cases) by touching a well-isolated colony with a straight wire and inoculating a tube of stock culture medium (either nutrient agar or semisolid trypticase soy agar). This single-colony pick, as well as any other single-colony picks done by other laboratories, may have selected a population that has lost the *Yersinia* virulence plasmid. Stock cultures were stored at room temperature without transfer, with a paraffin-coated cork or rubber stopper used to seal them. Beginning in 1985, we also made a frozen stock of the whole culture by harvesting cells from the heavy area of growth on a 1- to 3-day-old blood agar plate (25°C) into 10% skim milk. This was quick-frozen and stored at about −70°C. Unless otherwise specified, test were done at the Centers for Disease Control on fresh subcultures of stock cultures that had been stored at room temperature for 1 to 10 years.

Outbreaks. Table 2 lists 25 strains that were isolated during outbreak investigations. Some of these were nonpathogenic serotypes and were considered incidental to the outbreak itself. Outbreak 1 was an outbreak caused by *Y. enterocolitica* serotype O1,2,3 in chinchillas at several farms in California. Outbreak 2 occurred in a family group in Lee County, Ky., in 1974 (8). Outbreak 3 occurred in September and October 1975 in Oneida County, N.Y. (3), and was due to contaminated chocolate milk distributed as part of the school lunch program. Outbreak 4 occurred in 1981 and 1982 in Seattle, Wash., and was associated with eating tofu contaminated with *Y. enterocolitica* (17). A number of different serotypes, both pathogenic and nonpathogenic, were recovered during the outbreak investigations, but the majority from patients were serogroup O8 (data not shown). Outbreak 5 occurred during June and July 1982 in Tennessee, Arkansas, and Mississippi (18) and was due to *Y. enterocolitica* O13a,13b that contaminated pasteurized milk.

Strains from sporadic cases, old stock cultures, and other *Yersinia* species. Strains from sporadic cases, old stock cultures, and other *Yersinia* species (see Table 3) included 63 consecutive isolates that were referred to the Enteric Diseases Laboratory for one of our diagnostic services (serotyping, serodiagnosis, identification, etc.). Ten stock cultures of strains from the culture collection of the International *Yersinia* Centre at the Institut Pasteur, Paris, France, were also included, because these were more than 10 years old and presumably had been transferred many times, making it likely that they had lost the *Yersinia* virulence plasmid. Two cultures of serotype O9 isolated in Belgium were also included. Serotype O9 is rarely, if ever, isolated in the United States. For comparison, some other *Yersinia* species were also studied (see Table 4).

Serotyping. The O antigen was determined by microtube agglutination in 96-well plastic plates or by slide agglutination (3, 4). Absorbed sera were used when necessary. Strains that did not agglutinate in any of the sera used were coded NR, for no reaction. Strains that reacted in multiple sera were coded UND, for undefined. A few strains were rough and agglutinated in the saline control. All strains were defined as belonging to pathogenic or nonpathogenic serotypes on the basis of these serotyping results.

Tissue culture invasiveness. All 100 strains were tested at Stanford University for their ability to invade HEp-2 cells in culture (14). Briefly, about 2×10^7 bacterial cells were added to a monolayer of HEp-2 cells in a 24-well plate. After several incubation and handling steps, the HEp-2 cells were lysed to release intracellular bacteria, which were then recovered and counted on plating media. The study of Miller et al. (14) also gives the results for the strains listed in Tables 1 to 3 that were obtained with several DNA probes designed to measure chromosomal virulence. Cell invasion appears to be independent of the *Yersinia* virulence plasmid and is coded by genes on the chromosome of *Y. enterocolitica* (7, 14). Strains of pathogenic serotypes (as defined by serotyping) were positive for cell invasion, as measured by a tissue culture assay and by DNA probe assays (14), but strains of nonpathogenic serotypes were negative.

Evaluation of simple tests used to define pathogenic serotypes of *Y. enterocolitica*. Since antisera for typing clinical isolates of *Y. enterocolitica* are not generally available, several simple tests that can be used to identify pathogenic serotypes have been proposed. Table 1 summarizes the expected results for pathogenic and nonpathogenic serotypes in these tests.

Pyrazinamidase. The pyrazinamidase test was done as described by Kandolo and Wauters (10). Strains were inoculated over the entire slant of pyrazinamide agar, incubated at 25°C for 48 h, and tested with 1 ml of a freshly prepared 1% solution of ferrous ammonium sulfate. A positive pyrazinamidase reaction was indicated by a pink to brown color that developed on the slant.

Salicin-esculin. Salicin and esculin are both β-glucosides that vary in the chemical structure of the compound attached to D-glucose by a beta linkage. Both compounds are hydrolyzed by the enzyme β-glucosidase. Fermentation of salicin was determined in enteric fermentation base (catalog no. 1828-17-9; Difco Laboratories, Detroit, Mich.) with 1% salicin and Andrade indicator (all autoclaved at 121°C for 10 min). Esculin hydrolysis was determined in esculin broth (5 g of peptone, 1 g of KH_2PO_4, 5 g of esculin, 0.5 g of ferric citrate, and 10 ml of Andrade indicator with 990 ml of distilled water; all components were autoclaved at 121°C for 10 min). Strains were inoculated; incubated at 25°C; and read at days 1, 2, 3 (or 4 or 5), and 7.

D-Xylose fermentation. Strains of *Y. enterocolitica* serotype O3 do not ferment D-xylose (20). This is in contrast to other serotypes of this species and most of the other species of *Yersinia* that are xylose positive after 1 to 2 days of incubation at 25°C. Xylose fermentation was determined at 25°C in enteric fermentation base with 1% xylose (filter sterilized) and Andrade indicator.

CR-MOX agar. In 1989, Riley and Toma (16) described

TABLE 2. Reactions of 25 *Y. enterocolitica* strains isolated during outbreak investigations

Strain no.	Source	Outbreak no.	O group	Pathogenic serotype	Invasiveness (cell culture)	Pyrazinamidase	Salicin fermentation	Esculin hydrolysis	CR-MOX agar	Autoagglutination
9341-78 (JCF A1343)	Chinchilla	1	1,2,3	+	+	+ (Weak)	−	−	−	−
9286-78 (JCF A386)	Human (feces)	2	20	+	+	−	−	−	−	−
9287-78 (JCF A387)	Dog no. (feces)	2	20	+	+	−	−	−	−	−
9291-78 (JCF A400)	Human (cousin)	2	6	−	−	+	+	+	−	−
9292-78 (JCF A528)	Human (aunt)	2	22	−	−	+	−	−	−	−
9294-78 (JCF A0530)	Human (cousin)	2	22	−	−	+	−	−	−	−
9293-78 (JCF A529)	Human (grandfather)	2	Und[a]	−	−	+	+	+	−	−
JCF A2650	Human (throat)	3	8	+	+	−	−	−	−	−
9149-79 (JCF A2635)	Chocolate milk	3	8	+	ND[b]	−	−	−	−	−
9134-79 (JCF A3070)	1932 isolate	3	8	+	+	−	−	−	−	−
9141-79 (JCF A4186)	Human (sputum)	3	4,32	+	+	−	−	−	−	−
9142-79 (JCF A4182)	Human (feces)	3	6	−	−	+	+	+	−	−
1460-82 (A7525)	Human	4	8	+	+	−	−	−	−	−
1466-82 (A7443)	Human	4	8	+	+	−	−	−	−	−
1464-82 (A7534)	Human	4	4,32	+	+	−	−	−	+	+
1463-82 (A7532)	Human	4	21	+	+	−	−	−	−	−
1461-82 (A7527)	Human	4	6	−	−	+	+	+	−	−
1459-82 (A7523)	Human	4	7,13	−	−	+	+	+	−	−
1467-82 (A7458)	Human	4	7,13	−	−	+	+	+	−	−
1458-825 (A7513)	Tofu	4	Rough	−	−	+	+	+	−	−
1462-82 (A7528)	Human	4	Und	−	−	+	+	+	−	−
1176-82	Human (stool)	5	13a,13b	+	+	−	−	−	+	+
1211-82	Human (appendix)	5	13a,13b	+	+	−	−	−	−	−
1568-82	Human (stool)	5	13a,13b	+	+	−	−	−	−	−
8-83	Human (stool)	5	13a,13b	+	+	−	−	−	−	−

[a] Und, undetermined (no reaction in any of the O antisera).
[b] ND, Not done.

CR-MOX agar, which allows visualization of calcium-dependent growth and uptake of Congo red dye on the same plate. Cultures were streaked onto CR-MOX agar plates, incubated at 36°C, and observed for the presence of small red colonies at 24 and 48 h. Freshly isolated strains of pathogenic serotypes contain the *Yersinia* virulence plasmid and are CR-MOX positive (see Fig. 1). When the plates are kept at 36°C for several additional days, it is often possible to observe the development of a large, colorless colony from a small red colony.

Autoagglutination in broth that is temperature dependent. Cultures were inoculated into two tubes of MR-VP broth (Difco). One tube was incubated at 36°C and the other was incubated at 25°C. After 18 to 24 h, the tubes were observed for agglutination, with care taken not to shake or disturb the sediment at the bottom and along the sides of the tube. Strains of pathogenic serotypes that contain the *Yersinia* virulence plasmid agglutinate at 36°C but not 25°C. Strains that lack the virulence plasmid do not agglutinate at either temperature. Strains that agglutinated at both temperatures were considered "rough."

RESULTS AND DISCUSSION

Comparison of all results. Table 2 gives the results for individual strains isolated during outbreak investigations. Table 3 summarizes the results for strains from sporadic cases and stock cultures. Table 4 gives the results for the other species of *Yersinia*.

Pyrazinamidase test. The pyrizinamidase test was easy to do routinely. Most strains produced a strong pink-brown color or no color. However, three strains produced a slight amount of color (Table 5) that made it difficult to code them as positive or negative. These were read as "positive weak" and were considered positive in the tabulations. A negative pyrazinamidase test correctly identified 60 of 63 strains of pathogenic serotypes (95% sensitivity), and a positive pyrazinamidase test correctly identified 34 of the 37 strains of nonpathogenic serotypes (92% specificity). Thus, the pyrazinamidase test correctly identified 94% of the isolates. If the weak reactions had been coded negative, these percentages would have changed slightly. In their original description of the pyrazinamidase test, Kandolo and Wau-

TABLE 3. Reactions of *Y. enterocolitica* strains isolated from sporadic cases[a]

Serotype	No. studied	Pathogenic serotype	% Positive for:					
			Invasiveness (tissue culture)	Pyrazinamidase	Salicin fermentation	Esculin hydrolysis	CR-MOX (small red colonies)	Autoagglutination
1,2,3	1	Yes	100	100	0	0	100	100
2,3	1	Yes		0	0	0	0	0
3	16	Yes	100	0	0	0	38	19
4,32	2	Yes	100	0	0	0	0	0
5,27	6	Yes	100	17	0	0	33	0
8	9	Yes	100	0	0	0	11	11
9	3	Yes	100	0	0	0	100	100
13a,13b	3	Yes	100	0	0	0	0	0
18	1	Yes	100	0	0	0	0	0
20	4	Yes	100	0	0	0	0	0
21	2	Yes	100	0	0	0	50	50
5	3	No	0	100	100	100	0	0
6	5	No	0	100	100	100	0	0
7,13	2	No	0	100	100	100	0	0
7,19	1	No	0	0	100	100	0	0
7,8	3	No	0	67	100	100	0	0
10	2	No	0	50	50	50	0	0
22	1	No	0	100	100	100	0	0
27	4	No	0	100	100	100	0	0
28	1	No	0	100	100	100	0	0
Other[b]	5	No	0	100	100	100	0	0

[a] Includes 12 stock cultures: 3 of O9 and 1 each of O2,3; O3; O4,32; O5,27; O8; O18; O20; O21; and O27.
[b] Includes three rough strains and two strains that did not react with any of the antisera used.

ters (10) found 100% accuracy in differentiating pathogenic and nonpathogenic serotypes. However, Riley and Toma (16) noted that 5 of their 21 strains of *Y. enterocolitica* serotype O1,2,3 were pyrazinamidase positive, indicating a nonpathogenic serotype. We also noted that this serotype can be pyrazinamidase positive (Tables 2 and 3). Our data confirm that the pyrazinamidase test is a simple and reliable way of differentiating pathogenic and nonpathogenic serotypes (10, 16).

Salicin-esculin. There was 100% agreement between salicin fermentation and esculin hydrolysis (25°C, final reading at 2 days) for strains of *Y. enterocolitica* (Tables 2 and 3). Salicin fermentation was easy to read as positive or negative, but it was difficult to decide how much blackening was required for a positive esculin reaction. A negative reaction for salicin-esculin correctly identified all 63 strains of the pathogenic serotypes (100% sensitivity), and a positive result correctly identified 34 of 37 strains of nonpathogenic serotypes (92% specificity). Thus, salicin-esculin correctly identified 97% of the isolates, a percentage that was much better than expected. Wauters et al. (20) did not consider esculin hydrolysis to be a particularly useful test and cau-

TABLE 4. Reactions of six *Yersinia* species other than *Y. enterocolitica*

Species and strain no.	Source[a]	Invasiveness (tissue culture)	Pyrazinamidase	Salicin fermentation	Esculin hydrolysis	CR-MOX agar	Autoagglutination
Yersinia aldovae							
0669-83	Water	−	+	−	−	−	−
0670-83	Water	−	+	−	−	−	−
Yersinia frederiksenii							
1461-81		−	+	+	+	−	−
2581-77	Chocolate milk	−	+	+	+	−	−
Yersinia intermedia							
0031-83	Human urine	−	+	−	−	−	−
3208-86	Chocolate milk	−	+	+	+	−	−
Yersinia kristensenni							
1458-81		−	+	−	−	−	−
1465-82	Tofu	−	+	−	−	−	−
Yersinia rohdei							
3022-85	Dog stool	−	− (Weak)	−	−	−	−
3435-85	Human stool	−	− (Weak)	−	−	−	−
Yersinia "group ×1"							
9022-87		−	+ (Weak)	−	−	−	−
9023-87		−	+ (Weak)	−	−	−	−

[a] A blank indicates that the source is not known.

TABLE 5. Strains of *Y. enterocolitica* with results for pyrazinamidase differing from those for salicin-esculin

Serotype and strain	Serotype	Invasiveness (cell culture)	Pyrazinamidase	Salicin-esculin
Pathogenic				
2455-87	O1,2,3	+	+	−
9341-78	O1,2,3	+	+ (Weak)	−
3308-85	O5,27	+	+ (Weak)	−
Nonpathogenic				
3355-86	O7,8	−	−	+
0065-84	O7,19	−	−	+
1200-84	O10	−	−	+
9292-78	O22	−	+ (Weak)	−
9294-78	O22	−	+	−
2062-82	O10	−	+	−

tioned that a positive reaction is dependent on both temperature and time of incubation. We found that incubation at 25°C with a final reading at 2 days accurately differentiated pathogenic from nonpathogenic serotypes. The results for salicin-esculin at 36°C were not as helpful in identifying strains of nonpathogenic serotypes (data not shown) because positive reactions often occurred after the 48-h cutoff. It may not be necessary to do esculin hydrolysis routinely since it is more difficult to read and correlates 100% with salicin fermentation.

Correlation of pyrazinamidase with salicin-esculin. There was 91% agreement between the pyrazinamidase test and salicin-esculin (Table 5). Pyrazinamidase incorrectly identified strains of three pathogenic and three nonpathogenic serotypes. Salicin-esculin incorrectly identified strains of three nonpathogenic serotypes (Table 5). However, none of the nine strains were incorrectly identified by both the pyrazinamidase and the salicin-esculin tests. Both tests can be used routinely, and strains that provide conflicting results can be studied by other methods.

CR-MOX agar. Only 15 of the 63 strains of pathogenic serotypes produced small red colonies on CR-MOX agar. These results are in agreement with those of others (10, 12, 16, 19), who have noted that strains that have been stored or subcultured several times have often lost the *Yersinia* virulence plasmid. After 24 to 30 h of incubation, it is usually possible to identify a pathogenic serotype and determine the percentage of the population (colonies) that still contains the plasmid (Fig. 1).

Autoagglutination. Autoagglutination in MR-VP broth was easy to do and was easily adaptable to the daily routine since MR-VP broth is readily available in most laboratories. As with CR-MOX agar, old strains of pathogenic serotypes were usually negative. Only 8 of 63 strains were positive. Autoagglutination was usually clear and was easy to read; however, the tubes should be handled gently, because the agglutinated cells are easily resuspended, giving a uniform turbidity that would be read as negative. Although autoagglutination can be a useful test for fresh isolates, we no longer use it routinely, because CR-MOX agar provides the same information and has several advantages.

D-Xylose fermentation. All 16 strains of *Y. enterocolitica* serotype O3 were xylose negative, in contrast to other serotypes, which were uniformly positive. Thus, this simple test was extremely useful in quickly identifying this serotype, which is now the most common in the United States (2, 5, 13).

Reactions of other *Yersinia* species. Although the pyrazinamidase and salicin-esculin tests were useful in differentiating pathogenic and nonpathogenic serotypes of *Y. enterocolitica*, they should not be used until a strain has been identified to the species level. All strains of the other six *Yersinia* species were negative for tissue culture invasiveness and for the presence of the *Yersinia* virulence plasmid, as evidenced by their colonies on CR-MOX agar and lack of autoagglutination in MR-VP medium. These tests and many other reports indicate that strains of the six *Yersinia* species listed in Table 4 are not enteric pathogens (6, 7). However, four strains had very weak pyrazinamidase reactions and might be read as negative (Table 4). Nine strains were salicin-esculin negative, a property that is usually found in pathogenic serotypes of *Y. enterocolitica*. Thus, biochemical testing is essential for differentiating these other *Yersinia* species from *Y. enterocolitica*. Noble et al. (15) found that strains of some of the other *Yersinia* species had one or more properties perhaps suggestive of pathogenicity, but these results have not been confirmed. At present, there is no convincing evidence that strains of these other *Yersinia* species are enteric pathogens or are highly invasive for tissue. Further studies are needed to determine whether they have a role in human disease other than as opportunistic pathogens.

Until recently it has been very difficult for clinical and public health laboratories to determine the pathogenic potential of *Y. enterocolitica* strains isolated from feces (1, 9). Improvements in isolation methods for *Yersinia* such as CIN (cefsulodin-irgasan-novobiocin) medium and cold enrich-

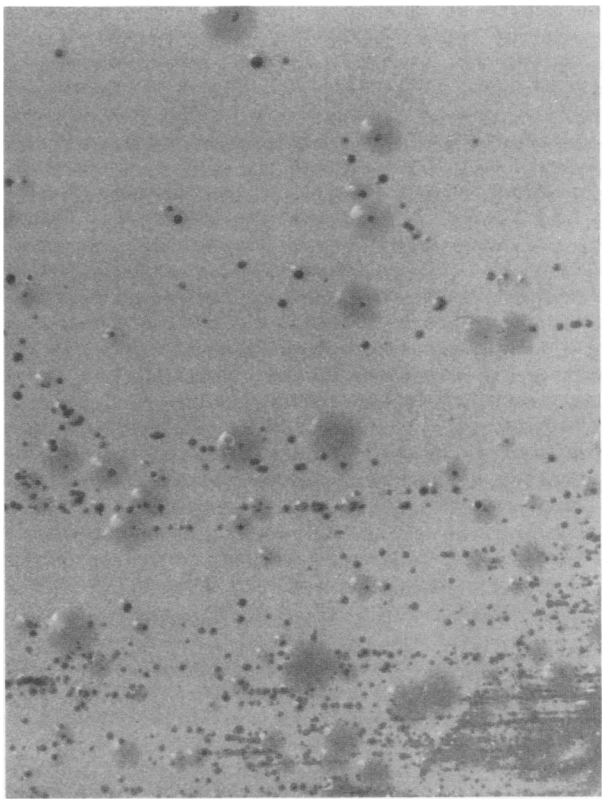

FIG. 1. A pathogenic serotype of *Y. enterocolitica* on CR-MOX agar (incubated at 36°C for 24 h). The colonies that appear black are dark red on the actual plate; some white colonies can be seen to be emerging from the tiny black colonies.

ment have made it possible to detect small numbers of *Yersinia* organisms mixed with normal enteric flora. Although this has greatly increased the isolation rate for *Yersinia* strains, many of these stool isolates will not be enteric pathogens. The first step in work with *Yersinia* strains is to identify the isolate to the species level, since no further testing is needed for species other than *Y. enterocolitica*. The four simple tests evaluated in the study described here can then be used to identify the isolate as a pathogenic or nonpathogenic serotype of *Y. enterocolitica*.

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