Isolation of *Yersinia* spp. from cases of diarrhoea in Iraqi infants and children

T.A. Kanan[1] and Z.A. Abdulla[1]

استفراد أنواع اليَرْسَنية من حالات الإسهال لدى الرُضَّع والأطفال في العراق

ذكرى ألكسان كنعان، زين العابدين عبد العزيز عبد الله

الخلاصة: شملت الدراسة جميع الأطفال الذين أحضروا إلى مستشفيين تعليميين في الموصل، في العراق خلال فترة تسعة أشهر لإصابتهم بالإسهال، بحثاً عن أنواع اليَرْسَنية في برازهم عن طريق الزرع المُغْنَى على البارد في درجة حرارة 4 سيلزيوس، لمدة 21 يوماً. وتم تعيين إمراضيَّة اليَرْسَنية المُسْتَفرَدة. وقد كانت أضداد اليَرْسَنية المعوية القولونية مرتفعة في الكشف السريع عن اليَرْسَنية في البراز. واستفرُدت أنواع اليَرْسَنية من براز أربعة أطفال فحسب؛ وحدد الباحثان هويتها على أنها اليَرْسَنية المعوية القولونية لدى ثلاثة منهم، واليَرْسَنية السلية الكاذبة لدى واحد منهم. وكان زرع الدم إيجابياً أيضاً في إحدى الحالات لليَرْسَنية المعوية القولونية. كما أجرى الباحثان اختبار حساسية الجراثيم للمضادات الحيوية في مستفردات اليَرْسَنية. وكشف الباحثان عن وجود تفاعل متصالب مصلي بين اليَرْسَنية السلية الكاذبة وبين السلمونيلة التيفية أو السلمونيلة نظيرة التيفية "بي"، وبين اليَرْسَنية المعوية القولونية والبروسيلة.

ABSTRACT All 250 children presenting with diarrhoea at 2 teaching hospitals in Mosul, Iraq over a 9-month period were studied for the presence of *Yersinia* spp. in stools by cold-enrichment culture at 4 °C for 21 days. Pathogenicity of the isolated *Yersinia* was determined. Antibodies to *Y. enterocolitica* were raised for rapid *Yersinia* detection in the stool. *Yersinia* spp. were isolated from the stools of only 4 patients; 3 isolates were identified as *Y. enterocolitica* and 1 was *Y. pseudotuberculosis*. The blood culture was also positive for *Y. enterocolitica* in 1 case. The antibiogram test for the isolated *Yersinia* was determined. Cross-reaction between *Y. pseudotuberculosis* and *Salmonella typhi* or *S. paratyphi* B, and between *Y. enterocolitica* and *Brucella* was detected serologically.

Isolement de *Yersinia* spp. à partir de cas de diarrhée chez des nourrissons et des enfants iraquiens

RÉSUMÉ Tous les 250 enfants qui ont présenté une diarrhée dans deux hôpitaux universitaires de Mosul (Iraq) pendant une période de 9 mois ont fait l'objet d'une recherche de *Yersinia* spp. dans les selles par le placement des cultures à une température de 4 ºC (technique d'enrichissement par le froid) pendant 21 jours. La pathogénicité de la bactérie *Yersinia* isolée a été établie. Des anticorps anti-*Y enterocolitica* ont été cultivés afin de permettre la détection rapide de *Yersinia* dans les selles. Des *Yersinia* spp. ont été isolées à partir des selles de 4 sujets seulement ; *Y. enterocolitica* a été identifiée dans 3 cas et *Y. pseudotuberculosis* dans 1. L'hémoculture était également positive à *Y. enterocolitica* dans 1 cas. On a déterminé l'antibiogramme pour la bactérie *Yersinia* isolée. La réaction croisée entre *Y. pseudotuberculosis* et *Salmonella typhi* ou *S. paratyphi* B, et entre *Y. enterocolitica* et *Brucella* a été détectée sérologiquement.

[1]*Department of Microbiology, College of Medicine, Mosul, Iraq (Correspondence to Z.A. Abdulla: pdrzainabdn@yahoo.com).*

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Introduction

Yersinia spp. are gram-negative rods or coccobacilli with bipolar staining that belong to the Enterobacteriaceae family [*1*]. Eleven species are known, but only 3 are important human pathogens; *Y. enterocolitica* is the most common of these, while *Y. pseudotuberculosis* is less frequent, and *Y. pestis* is rare [*2*].

Yersiniosis is a clinical condition caused by infection with low- and high-pathogenic species of *Yersinia*: *Y. enterocolitica* and *Y. pseudotuberculosis* [*3,4*]. The disease is characterized by symptoms of gastroenteritis and/or vomiting with abdominal pain. *Yersinia* infection ranges from asymptomatic hosts to patients with life-threatening sepsis, especially in children [*1*]. Several syndromes are associated with *Y. enterocolitica* infection in children, including enterocolitis, pseudoappendicitis syndrome, extraintestinal infections, bacteraemia and Izumi fever.

Transmission is primarily via ingestion of contaminated foods, water and milk or ingestion of uncooked meat products, especially pork [*5*]. The majority of cases of enterocolitis are seen in children aged 1–4 years [*6,7*]. Moreover, these infections show a modest predilection for males, with male to female ratio of 1.7:1.

Yersiniosis is a rare disease in Muslim countries due to the scarcity of pork consumption. The incidence of yersiniosis is reported to be 10%–30% in European countries and 0.06%–2% in Muslim ones [*8,9*]. The weather is another factor affecting the growth and transmission of *Yersinia*. A cold climate facilitates pathogenesis; this is encountered most of the year in European countries, whereas in temperate countries such as Iraq it is mainly in the winter months.

The aim of the present study was to investigate the role of *Yersinia* as a cause of diarrhoea among infants and children in the Iraqi community since no local data about the subject are available.

Methods

Patients

The study sample was all 250 infants and children suffering from diarrhoea who were admitted to the Department of Paediatrics in Ibn Alatheer and Ibn Seena teaching hospitals, Mosul, Iraq from October 2003 to June 2004. The duration of diarrhoea ranged from 1 day to 1 month, but 188 cases (75%) had duration of < 5 days. Besides diarrhoea, the patients presented with fever in 195 cases (78%) and vomiting in 188 cases (75%). The patients were 139 males (55.6%) and 111 females (44.4%), with mean age 13.9 [standard deviation (SD) 15.2] months, range 12 days to 12 years.

The cases were subdivided into 2 groups according to the method used for identification. A subsample of 100 patients underwent full bacteriological identification from the stool and blood samples to identify the concomitant bacteria encountered in *Yersinia* positive or negative cases and to study the survival of *Yersinia* and other bacteria in cold enrichment. The whole group of 250 patients underwent rapid identification of *Yersinia* from the stool samples only to determine the highest possible number of cases of yersiniosis.

Stool culture

Ordinary culture was done for the subsample of 100 patients. Stool samples were cultured directly on MacConkey agar (Oxoid, UK) and Salmonella–Shigella agar (Himedia, India) and incubated for 24 hours at 25 ºC.

Cold enrichment was done for all cases. Faecal samples were cultured in phosphate

buffered saline, incubated for 3 weeks at 4 ºC and subcultured on MacConkey and Salmonella–Shigella agar every 7 days. The plates were incubated at 25 ºC for 24–48 hours according to Sonnenwirth and Jarett [*8*].

Blood culture

The blood for culture was taken before the administration of antibiotics. The skin at the vein puncture was prepared using bactericidal disinfectant (2% solution of iodine and 70% alcohol). The blood was mixed with 10 times its volume of brain–heart infusion (BHI) broth in bottles. The cultures were incubated at 25 ºC for 15 days. Each sample was subcultured on blood and MacConkey agars after 7 and 15 days of incubation. Positive cultures showed turbidity; negative ones showed a layer of sediment of red blood cells covered by pale yellow transparent broth.

Pathogenicity tests

The isolated *Yersinia* spp. were subjected to 3 different pathogenicity tests to differentiate between the pathogenic and the non-pathogenic strains: the autoagglutination test, the crystal violet binding test and animal inoculation.

Auto-agglutination test

Two tubes of glucose–phosphate–peptone water were inoculated with a colony of *Yersinia*; 1 tube was incubated at 25 ºC and 1 tube at 35–37 ºC [*2*].

Crystal violet binding test

Colonies of *Yersinia* were cultured in BHI broth (Biokit, Spain) for 18 hours at 22–26 ºC. Subculture was done on 2 BHI agar plates (Oxoid, UK); 1 plate was incubated at 25 ºC and 1 plate at 37 ºC for 30 hours [*2*].

Animal inoculation

Three animal pathogenicity tests were done to determine the pathogenicity of *Yersinia* and their systemic and histopathological effects.

Intraperitoneal infection of rabbits was done by culturing *Y. enterocolitica* and *Y. pseudotuberculosis* in nutrient broth for 24 hours, serial dilution was done in normal saline and 3 rabbits were injected intraperitoneally with 3×10^7 cells/mL. One rabbit was injected with *Y. pseudotuberculosis* and 2 with *Y. enterocolitica*.

Mouse infection was done by culturing the bacteria in BHI broth for 24 hours at 25 ºC, and serial dilutions were done in physiological saline. A group of 6–8-week-old white mice was injected intraperitoneally with 0.1 mL and 0.2 mL in dilutions of 3×10^6 cells/mL and 3×10^7 cells/mL. On the 5th post-infection day, the mice were killed and the liver, spleen, intestine and mesenteric lymph nodes were extracted and sent for the histopathological study at the Department of Histopathology, College of Medicine, Mosul, Iraq.

Detection of enterotoxins of the isolated *Yersinia* was assessed in infant mice. The bacteria were cultured by shaking in trypticase–soy broth (Oxoid, UK) containing 0.6% (wt./vol.) yeast extract at 28 ºC for 48 hours. Bacterial cells were removed by cold centrifugation, and 0.1 mL of the supernatant was administered orally to 2–3-day-old mice in a dilution of 3×10^6 cells/mL. After 2 hours, the mice were killed and through abdominal exploration, swelling of the intestines of the infected mice was noted. The mean ratio of intestinal weight to the remaining body weight was calculated. Ratios greater than 0.08 were considered indicative of enterotoxin production according to Grant et al. [*9*].

Anti-*Yersinia* antibody production

The specificity of the raised anti-*Yersinia* antibodies was tested using *Yersinia* and other microorganisms that possibly cross-react.

Antibody was raised in 6–8-week-old mice. Three mice were given repeated injections *of Y. enterocolitica*, the doses varied from 0.1–0.2 mL in a dilution of 3×10^6 bacterial cells/mL. These injections were given in 5 doses, 4 intraperitoneally and 1 intravenously with 6 days interval between the doses. The animals were bled and the serum was separated and frozen at –20 ºC until use. Detection of anti-*Yersinia* antibodies in serum was done by mixing a drop of serum taken from infected adult mice with a colony taken from a pure culture of *Y. enterocolitica*, and obvious agglutination was seen in less than 1 minute.

Bacterial suspensions of 4 common genera of microorganisms that have possible cross-reaction with *Yersinia* were used to test the raised anti-*Yersinia* antibodies. These were *E. coli*, *Salmonella*, *Klebsiella* and *Proteus* spp.

Serological tests

The following serological tests were done for the subsample of 100 patients, according to the methods of Osman et al. [*10*].

- Rapid slide agglutination test (Widal test)
- *Brucella* agglutination test (rose–Bengal test)
- 2-mercaptoethanol (2ME) test for both Widal and *Brucella* agglutination tests.

Antimicrobial sensitivity test

Selected colonies were suspended in 0.85% saline solution to achieve a turbid suspension and spread on Muller–Hinton agar plates (Oxoid, UK) using cotton swabs. The antibiogram was done for both *Yersinia* and non-*Yersinia* isolates.

Results

Stool culture

The cold enrichment culture was done for all 250 patients studied. The total number of *Yersinia* isolates from the stool culture of all patients studied was 4 (1.6%); 3 of these isolates were *Y. enterocolitica* and 1 was *Y. pseudotuberculosis* (Table 1). The 3 isolates of *Y. enterocolitica* were recovered from children aged 1–11 months, while the 1 strain of *Y. pseudotuberculosis* was isolated from a 5-year-old child.

Yersinia was not isolated by the 7 days of cold enrichment, but 3 isolates appeared by 14 days and 1 isolate by 21 days (Table 2).

Blood culture

Blood culture for the subsample of 100 cases was positive for *Y. enterocolitica* in 1 case (1%) only, an infant aged 1 month. This patient also had positive stool culture for the same microorganism. The stool and the blood isolates showed similar morphological and biochemical characteristics of *Y. enterocolitica* (Table 3).

Pathogenicity tests

Auto-agglutination tests

In auto-agglutination tests, after 24 hours the tube incubated at 25 ºC showed some turbidity of bacterial growth, while the tube incubated at 37 ºC showed agglutination of

Table 1 **Incidence of *Yersinia* spp. isolated from the stool culture of 250 patients**

Species	No. of isolates	% of patients (*n* = 250)	% of isolates (*n* = 4)	Incidence (per 1000 patients)
Y. enterocolitica	3	1.2	75	12
Y. pseudotuberculosis	1	0.4	25	4
Total	4	1.6	100	16

Table 2 **Bacteria isolated from stool culture by direct and cold-enrichment technique in 100 patients as a proportion of the number of patients and number of isolates**

Bacteria	Ordinary culture		Cold-enrichment culture						Reduction factor[e]
			7 days		14 days		21 days		
	Patients (%) (*n* = 100)	Isolates (%) (*n* = 174)	Patients (%) (*n* = 100)	Isolates (%) (*n* = 114)	Patients (%) (*n* = 100)	Isolates (%) (*n* = 91)	Patients (%) (*n* = 100)	Isolates (%) (*n* = 79)	
Escherichia coli	61.0	35.0	40.0	35.0	28.0	30.0	25.0	31.6	2.4
Enterobacter spp.[a]	30.0	17.2	20.0	17.5	18.0	19.7	16.0	20.0	1.9
Klebsiella spp.[b]	20.0	11.5	12.0	10.5	10.0	10.9	9.0	11.3	2.2
Proteus spp.[c]	18.0	10.3	10.0	8.7	7.0	7.7	5.0	6.3	3.6
Citrobacter spp.[d]	14.0	8.0	6.0	5.2	2.0	2.2	2.0	2.5	7.0
Pseudomonas aeruginosa	10.0	5.7	10.0	8.7	10.0	10.9	10.0	12.6	1.0
Shigella spp.	3.0	1.7	2.0	1.7	2.0	2.2	2.0	2.5	1.5
Salmonella spp.	3.0	1.7	5.0	4.3	2.0	2.2	1.0	1.3	3.0
Yersinia spp.	0.0	0.0	0.0	0.0	2.0	2.2	1.0	1.3	0
Y. enterocolitica	0.0	0.0	0.0	0.0	1.0	1.1	0.0	0.0	0
Y. pseudotuberculosis	0.0	0.0	0.0	0.0	1.0	1.1	1.0	1.3	0
Alcaligenes faecalis	1.0	0.8	1.0	0.9	2.0	2.2	2.0	2.5	0.5
Aeromonas spp.	2.0	1.2	3.0	2.6	3.0	3.2	3.0	3.8	0.7
Staphylococcus aureus	4.0	2.3	2.0	1.7	2.0	2.2	1.0	1.3	4.0
Streptococcus faecalis	5.0	2.8	2.0	1.7	2.0	2.2	1.0	1.3	5.0
Candida albicans	3.0	1.7	1.0	0.9	1.0	1.1	1.0	1.3	3.0
Total	174.0	100.0	114.0	100.0	91.0	100.0	79.0	100.0	2.2

[a] *Includes* Enterobacter hafnia, E. agglomerans *and* E. cloacae; [b] *Includes K*lebsiella pneumoniae *and* K. ozaenae; [c] *Includes* Proteus vulgaris *and* P. mirabilis; [d] *Includes C*itrobacter freundii *and* C. diversus.

[e] *Reduction factor is the ratio between percentage of growth in ordinary culture (1st column) and cold enrichment growth after 21 days of incubation (7th column).*

bacteria along the walls and the bottom of the tube with clear supernatant fluid.

Crystal violet binding test

The crystal violet binding test showed that colonies grown at 37 ºC bound to the stain, while colonies grown at 25 ºC did not.

Animal pathogenicity tests

In the animal pathogenicity tests the rabbit that was given *Y. pseudotuberculosis* died after the 3rd injection (12 days), the remaining 2 rabbits that were given *Y. enterocolitica* died after the 4th injection (18 days).

The hematoxylin–eosin-stained sections of all the tissues taken from the group of animals injected with 0.1 mL and 0.2 mL of 3×10^6 bacterial cells/mL showed no histopathological changes. However, in the group of animals injected with 0.2 mL of 3×10^7 bacterial cells/mL, their tissues showed chronic inflammatory cell infiltrate, mainly lymphocytes. These changes were seen in the intestine and the mesenteric lymph nodes. Granulomatous changes were seen in the lungs. No inflammatory changes were noted either in the liver or the spleen.

Enterotoxin production was positive in infant mice with ratios of intestine:total body weight of 0.08 and 0.083.

Table 3 **Bacteria isolated from blood culture of 100 patients**

Bacteria	Patients (*n* = 100)		Isolates (*n* = 12)
	No.	%	%
Staphylococcus aureus	5	5.0	41.7
Escherichia coli	4	4.0	33.3
Enterobacter hafnia	1	1.0	8.3
Yersinia enterocolitica	1	1.0	8.3
Listeria monocytogenes	1	1.0	8.3
Total	12	12.0	100.0

Anti-*Yersinia* antibody production

None of the bacterial suspensions tested (*E. coli*, *Salmonella*, *Klebsiella* or *Proteus* spp.) showed macroscopical agglutination. The antibody reacted only with *Y. enterocolitica*, as a positive result was noted when the stool of a yersiniosis patient was mixed with the antiserum.

Serological tests

The Widal agglutination test was positive in 6 out of 100 patients tested. Among these positive cases, only 1 had *Y. pseudotuberculosis* infection. This microorganism was only identified from the stool and not from the blood. This patient had anti-O antibodies against *Salmonella typhi* (1/160) and *Salmonella paratyphi* B (1/320) without anti-H antibodies. The 2ME Widal test of this patient revealed negative agglutination for anti-O antibodies of both *S. typhi* and *S. paratyphi* B.

The *Brucella* agglutination test was positive in 1 patient only (1%) with a titre of 1/160. This patient had positive stool and blood cultures for *Y. enterocolitica* and negative 2ME *Brucella* agglutination test (which detects active recent infection).

Antimicrobial sensitivity tests

In the antibiogram tests, 4 isolates of *Y. enterocolitica* and 1 of *Y. pseudotuberculosis* showed full sensitivity to gentamicin, cefotaxime, chloramphenicol, amikacin, ciprofloxacin and norfloxacin. Two of the 3 *Y. enterocolitica* strains were sensitive to nalidixic acid and tetracycline, while *Y. pseudotuberculosis* was also sensitive to these 2 drugs (Table 4).

All *Yersinia* spp. isolated from the stool were resistant to rifampin, imipenem and ceftazidime. *Y. pseudotuberculosis* showed an intermediate sensitivity to amoxycillin and cephalexin, while *Y. enterocolitica* isolates were resistant to these 2 drugs.

Discussion

In this study *Y. enterocolitica* was isolated from 3 children aged 1–11 months, while *Y. pseudotuberculosis* was isolated from a 5-year-old child. These results are in agreement with previous studies in which *Y. enterocolitica* infection was found in infants and young children whereas *Y. pseudotuberculosis* infected older children aged 5–15 years [*2,11*].

Y. enterocolitica was isolated from the blood of an infant aged 1 month, who also had a positive stool culture for the same microorganism. This result is supported by previous studies which reported that concomitant bacteraemia was seen in 20%–30% of infants younger than 3 months infected with this microorganism [*12*]. However, we could not isolate *Y. pseudotuberculosis* from the blood of any cases although it was found in the stool of an older child (aged 5 years).

The pathogenicity tests, the auto-agglutination test, crystal violet binding test and *Yersinia* inoculation in animals indicated that all *Yersinia* isolates were pathogenic and the pathogenicity of *Y. pseudotuberculosis* was greater than that of *Y. enterocolitica* as determined by animal inoculation. This finding is in accordance with many previous studies [*1,4*]. Also Delor and Cornelis considered that the *Yersinia* enterotoxin was the cause of diarrhoea in young rabbits and consequently was the major factor involved in the *Y. enterocolitica*-associated diarrhoea in young children. [*13*].

The effect of *Y. enterocolitica* isolates injected into adult mice on the lymph nodes, intestine and lungs indicated that the primary site of *Yersinia* infection and multiplication was the lymphatic tissue. This result is in keeping with Grant et al. [*9*] and Iwobi et al. [*4*] who found that the Peyer's patches

Table 4 **Antibiogram of the isolated *Yersinia* strains**

Antibiotic	**No. of sensitive strains**			
		Y. enterocolitica		***Y. pseudotuberculosis***
	All isolates (*n* = 4)	**Stool (*n* = 3)**	**Blood (*n* = 1)**	**Stool (*n* = 1)**
Amoxicillin	0	0	0	1
Cefotaxime	4	3	1	1
Cephalexin	0	0	0	1
Ceftazidime	0	0	0	0
Imipenem	0	0	0	0
Gentamicin	4	3	1	1
Chloramphenicol	4	3	1	1
Tetracycline[a]	3	2	1	1
Nalidixic acid	3	2	1	1
Trimethoprim	1	1	0	1
Rifampin	0	0	0	0
Amikacin	4	3	1	1
Netilimicin	1	1	0	0
Piperacillin	1	1	0	0
Ciprofloxacin[a]	4	3	1	1
Norfloxacin[a]	4	3	1	1

[a]*These antibiotics are not suitable for use in children.*

of the intestine were the primary sites for infection with *Yersinia*. Moreover, enterotoxin production was positive in infant mice with ratios of intestine:total body weight of 0.08 and 0.083. This result is in accordance with Nunes and Ricciardi [*14*] and Grant et al. [*9*] who considered intestinal swelling and ratios of 0.08 and above as indicators of enterotoxin production.

The isolation of *Yersinia* from the stool requires at least 2–3 weeks of cold enrichment culturing which renders this method laborious for routine work. Therefore, it is recommended that culturing is done by more practical methods such as serological tests using the specific antibody agglutination test.

The specificity of the raised anti-*Yersinia* antibody was tested using *Yersinia* and other microorganisms that possibly cross-react with it: the antibody reacted only with *Y. enterocolitica*. Consequently, this serological method was used as a possible alternative test for the diagnosis of yersiniosis. We showed that *Y. enterocolitica* could cross-react with *Brucella* and yield a false-positive rose–Bengal test in *Brucella* non-infected patients. Many researchers have demonstrated the cross-reaction between *Y. enterocolitica* serotype 0:9, and *Brucella abortus* and *B. melitensis* [*15,16*]. It is possible that *Y. enterocolitica* isolated in the present study may belong to serotype 0:9 as it is the main serotype that can cross-react with *Brucella*. Cross-reaction was also found between *Y. pseudotuberculosis* and group B and D *Salmonella* spp. This is in keeping with other studies [*12,17*].

The most effective drugs (full sensitivity) in treating yersiniosis were gentamicin, cefotaxime, chloramphenicol and tetracycline. This result is in agreement with that of Tzelepi et al. [*18*]. These antibiotics were effective against *Yersinia* isolated from both the stool and the blood. Also, the full resistance of *Y. enterocolitica* to both amoxicillin and cephalexin agrees with Tzelepi et al., who mentioned that *Y. enterocolitica* produces chromosomally determined β-lactamases that cause resistance to these 2 antibiotics [*18*]. The sensitivity of the single isolate of *Y. pseudotuberculosis* to amoxycillin and cephalexin contradicts Morris's opinion that this microorganism was resistant to these antibiotics [*14*]. However, these results are in accordance with reports by Campbell and Dennis [*11*]. Such discrepancies might be attributed to strain or biotype variations.

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