1. Burger, J. W., *J. Exp. Zool.*, 1939, **81**, 333–341.
2. Bissonnette, T. H., *Physiol. Zool.*, 1931, **4**, 542–574.
3. Bentley, G. E., Goldsmith, A. R., Dawson, A., Briggs, C. and Pemberton, M., *J. Biol. Rhythms*, 1998, **13**, 148–158.
4. Benoit, J., *Ann. NY Acad. Sci.*, 1964, **117**, 204–216.
5. Oishi, T. and Lauber, J. K., *Am. J. Physiol.*, 1973, **225**, 880–886.
6. Kumar, V. and Rani, S., *Physiol. Behav.*, 1996, **60**, 625–631.
7. Rani, S. and Kumar, V., *ibid.*, 2000, **69**, 277–283.
8. Kumar, V., Jain, N., Singh, B. P. and Kumar, B. S., *Reprod. Nutr. Dev.*, 1993, **33**, 143–150.
9. Tewary, P. D. and Kumar, V., *J. Comp. Physiol. B*, 1981, **144**, 201–203.
10. Tewary, P. D. and Kumar, V., *Curr. Sci.*, 1984, **53**, 113–114.
11. Kumar, V., Jain, N. and Follett, B. K., *J. Comp. Physiol. A*, 1996, **179**, 59–64.
12. Kumar, V. and Tewary, P. D., *ibis*, 1983, **125**, 305–312.
13. Kumar, V. and Rani, S., *Curr. Sci.*, 1999, **77**, 283–285.
14. Seliger, H. H. and McElroy, W. D. (eds), in *Light: Physical and Biological Action*, Academic Press, New York, 1965.
15. Hartwig, H. G. and Veen, T. van, *J. Comp. Physiol. A*, 1979, **130**, 277–282.
16. Joshi, D. and Chandrashekaran, M. K., *ibid.*, 1985, **156**, 189–198.
17. Boulos, Z., *Physiol. Behav.*, 1995, **57**, 1025–1033.

ACKNOWLEDGEMENTS. We thank the anonymous reviewer(s) for helpful comments on the earlier version of the manuscript. Financial support from the Council of Scientific and Industrial Research, New Delhi, through a research grant to V.K. is acknowledged.

Received 29 February 2000; revised accepted 5 June 2000

Isolation of *Yersinia enterocolitica* and *Yersinia intermedia* from wastewaters and their biochemical and serological characteristics

Indrajit Sinha, Indu Choudhary and J. S. Virdi*

Microbial Pathogenicity Laboratory, Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110 021, India

***Yersinia enterocolitica*, an important food- and water-borne enteric pathogen was isolated from wastewater samples collected in and around the national capital territory of Delhi. All *Y. enterocolitica* isolates belonged to biotype 1A. Among these, O:6,30–6,31 was the predominant serotype. Serotypes O:10-34; O:6,31; O:15; O:41, 42 and O:41, 43 were also isolated. Except for two non-agglutinable isolates belonging to biotype 1 and 2, all *Y. intermedia* isolated were of serotype O:40 (biotype 4). Majority of the *Y. intermedia* isolates were rhamnose negative, representing the rare biotype. This study reports occurrence of *Y. enterocolitica* and *Y. intermedia* in wastewaters in India and highlights the need for further surveillance studies on *Y. enterocolitica* in understanding the global epidemiology of such emerging pathogens.**

YERSINIA enterocolitica, an important food- and water-borne gastrointestinal agent is regarded as an emerging pathogen worldwide[1]. In India, the first outbreak of gastroenteritis due to *Y. enterocolitica* was reported recently from the North Arcot district of Tamil Nadu and the organism was isolated from well water used to dilute the buttermilk consumed by the affected individuals[2]. Earlier, in India, *Y. enterocolitica* has been isolated from the stools of diarrhoeic patients[3–5], pigs[6,7], pork[8] and fresh buffalo milk[9]. Serological evidence of porcine yersiniosis has been reported from Bangalore[10]. In an another study however, *Y. enterocolitica* could not be isolated from diarrhoeic stools of farm animals, namely cows and buffaloes[11]. There has been no systematic study on the prevalence of water-borne *Y. enterocolitica* in India. This study reports the occurrence of *Y. enterocolitica* and *Y. intermedia* in wastewater from Delhi. Data on the serological and biochemical characteristics of these isolates are also presented.

The study was carried out in the national capital territory of Delhi. Wastewater samples were collected over a period of one and half years, from five sewage treatment plants (50 samples), three major drains (15 samples) and from the river Yamuna (7 samples) (Figure 1). The sewage treatment plants located at Okhla (capacity 150 million gallons per day, MGD), Keshopur (86

Figure 1. Map showing study area and sampling sites.

*For correspondence. (e-mail: micro@dusc.ernet.in)

MGD), Rithala (48 MGD) and Kondli (12 MGD) have primary and secondary facilities for treatment of wastewaters, while the one at Timarpur (15 MGD) consists of a series of oxidation ponds. Among the three drains from which wastewater samples were collected, Najafgarh drain is the largest. It originates from Haryana and flows through a larger part of west Delhi and finally joins Yamuna at the Wazirabad Bridge. The Shahdara drain collects wastewater from the residential colonies in this area and joins Yamuna. The Hindan drain carries wastewater from a part of eastern Delhi to Yamuna.

Water samples were collected in sterilized screw-capped glass bottles without any additive. These were transported to the laboratory at 4°C and processed within 6 h of their collection. 25 to 30 ml of the sample was inoculated into 10-fold volume of phosphate-buffered saline containing sorbitol and bile salts[12]. It was kept at 4°C for 3 weeks. After this period, a loopful of the cold-enriched sample was treated with 0.5% KOH in 0.5% sodium chloride for 30 s (ref. 13). Cold-enriched and KOH-treated samples were plated on CIN (Cefsulodin – Irgasan – Novobiocin)[14] and MacConkey agar medium (HiMedia, Mumbai). The plates were incubated at 25 to 26°C for 24 to 48 h. Colonies having features of characteristic bull's eye morphology with deep red centres and white to translucent periphery on CIN agar, and flat non-lactose fermenting (NLF) growth on MacConkey agar were selected. These suspected isolates were subjected to detailed biochemical characterization[15]. Species identification was done by fermentation of rhamnose, melibiose, α-methyl-D-glucoside, raffinose and sucrose. Isolates identified as belonging to the genus *Yersinia* were sent to Central Public Health Laboratory, Colindale, London or Institut Pasteur, Paris, France for confirmation and serotyping.

Yersinia was isolated from twenty-two of the fifty sewage effluent samples collected. Of the total positive samples, 11 were positive for *Y. enterocolitica*, 8 for *Y. intermedia* and 3 for both *Y. enterocolitica* and *Y. intermedia*. None of the samples collected from drains or the river yielded *Y. enterocolitica* or *Y. intermedia*. On CIN agar, the colonies of the isolates were smooth, convex, glistening and showed characteristic bull's eye appearance with a deep red centre having white to translucent periphery with entire margins (Figure 2). When examined under a stereomicroscope (10 ×) the colonies appeared raised with sharply defined red centres. On MacConkey agar the colonies were invariably smaller than those on the CIN agar and were NLF, flat and without entire margins. Microscopically, all isolates were gram-negative rods, motile at 25°C and non-motile at 37°C.

All *Y. enterocolitica* were urease positive, and produced alkaline (pink) slant and acid (yellow) butt without the production of H_2S or gas on Kligler iron agar slants. This indicated that the isolates characteristically utilized glucose but failed to utilize lactose. All the strains showed negative reactions for lysine decarboxylase, arginine dihydrolase and phenylalanine deaminase, and positive reaction for ornithine decarboxylase. All the strains failed to utilize malonate, produced indole (except for 6 isolates) and showed minimal growth in KCN. The isolates were negative for rhamnose, melibiose, α-methyl-D-glucoside and raffinose. Most *Y. intermedia* isolates showed biochemical reactions characteristic of typical *Y. intermedia*, i.e. positive for rhamnose, melibiose and raffinose. However, 6 of these (K/Y/3; K/Y/28; K/Y/29, K/Y/30, R/Y/59 and O/Y/66) failed to utilize rhamnose.

Serotyping and biotyping data of the isolates are given in Table 1. Strains of *Y. enterocolitica* belonged to serotype O:6,30-6,31 (7 isolates), O:10-34 (5 isolates) and one each to O:6,31, O:15; O:41,42 and O:41,43. Three isolates were untypable (non-agglutinable or NAG). All *Y. enterocolitica* isolates were of biotype IA. All *Y. intermedia* isolates, except the three non-agglutinable, were of serotype O:40. These belonged to biotype 4 (10 isolates), biotype 1 (1 isolate) and biotype 2 (1 isolate).

Although, in India, *Y. enterocolitica* has been isolated from various sources, this report represents the first isolation of this organism from wastewaters. To the best of our knowledge, this also represents the largest number of *Y. enterocolitica* and *Y. intermedia* isolated from the Indian subcontinent. Earlier, serotypes O:1,2a,3, O:3, O:4, O:9 and O:9,16 were isolated from human diarrhoeic stools[3–5] and pigs[7], O:7,8-8-8,19 from pork[8] while O:5 and O:22 were isolated from raw milk[16] and sugarcane juice[16] respectively. *Y. enterocolitica* isolated from well water and implicated in the first outbreak of gastroenteritis due to this organism in India belonged

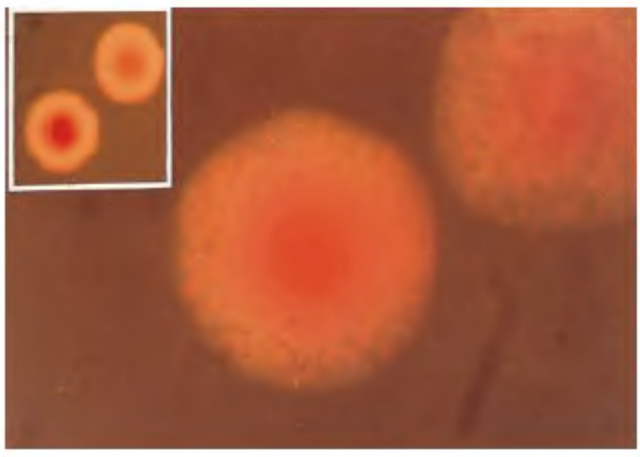

Figure 2. Characteristic bull's eye colony of *Y. enterocolitica* on Cefsulodin–Irgasan–Novobicocin (CIN) agar as seen under stereomicroscope (10×). (Inset) The same under lower magnification.

Table 1. Species, serotypes and biotypes of *Yersinia* spp. isolated from wastewaters

Isolate	Date	Species	Serotype	Biotype	Reference lab. no.
R/Y/2*	25.06.97	*Y. enterocolitica*	O:41,43	1A	E1281550
K/Y/3*	23.06.97	*Y. intermedia*	NAG	4	E1281560
Ko/Y/11	01.07.97	*Y. enterocolitica*	NAG	1A	E1281570
Ko/Y/12*	01.07.97	*Y. enterocolitica*	O:15	1A	E1281580
Ko/Y/16	01.07.97	*Y. enterocolitica*	NAG	1A	E1281590
Ko/Y/17	01.07.97	*Y. enterocolitica*	NAG	1A	E1281600
K/Y/30*	11.08.97	*Y. intermedia*	O:40	4	IP26142
K/Y/31	11.08.97	*Y. intermedia*	O:40	4	IP26143
T/Y/33	22.08.97	*Y. intermedia*	NAG	1	IP26259
T/Y/34*	22.08.97	*Y. enterocolitica*	O:6,30-6,31	1A	IP26144
R/Y/36	22.08.97	*Y. enterocolitica*	O:6,30-6,31	1A	IP26145
O/Y/43	22.08.97	*Y. intermedia*	O:40	4	IP26146
O/Y/44	22.08.97	*Y. enterocolitica*	O:6,31	1A	IP26260
T/Y/48	22.08.97	*Y. enterocolitica*	O:10-34	1A	IP26261
T/Y/53	04.07.97	*Y. intermedia*	O:40	4	IP26262
T/Y/54*	04.07.97	*Y. enterocolitica*	O:10-34	1A	IP26147
T/Y/55	05.10.98	*Y. intermedia*	O:40	4	IP26304
R/Y/56	05.10.98	*Y. enterocolitica*	O:10-34	1A	IP26305
Ko/Y/57	05.10.98	*Y. intermedia*	NAG	2	IP26306
R/Y/59	12.10.98	*Y. intermedia*	O:40	4	IP26307
O/Y/60	19.10.98	*Y. intermedia*	O:40	4	IP26308
O/Y/61	26.10.98	*Y. enterocolitica*	O:10-34	1A	IP26309
K/Y/62	31.10.98	*Y. enterocolitica*	O:6,30-6,31	1A	IP26310
Ko/Y/63	31.10.98	*Y. enterocolitica*	O:6,30-6,31	1A	IP26311
K/Y/64	07.11.98	*Y. enterocolitica*	O:6,30-6,31	1A	IP26312
T/Y/65	07.11.98	*Y. intermedia*	O:40	4	IP26318
O/Y/66	07.11.98	*Y. intermddia*	O:40	4	IP26313
R/Y/67	14.11.98	*Y. enterocolitica*	O:10-34	1A	IP26314
K/Y/68	16.11.98	*Y. enterocolitica*	O:6,30-6,31	1A	IP26315
Ko/Y/69	16.11.98	*Y. enterocolitica*	O:41,42	1A	IP26316
O/Y/70	16.11.98	*Y. enterocolitica*	O:6,30-6,31	1A	IP26317

Strains marked with asterisk (*) have been deposited with Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (Chandigarh) and have been assigned numbers as: MTCC 3099 (R/Y/2); MTCC 3101 (K/Y/3); MTCC 3100 (Ko/Y/12); MTCC 3235 (K/Y/30); MTCC 3236 (T/Y/34) and MTCC 3233 (T/Y/54); NAG, non-agglutinable; E, Laboratory of Enteric Pathogens, CPHL, London; IP, Institut Pasteur, Paris, France.

to serotype O:3 (biotype 4)[2]. The serotypes namely O:6, 30-6,31, O:6,31, O:10-34, O:15, O:41,42 and O:41,43 as reported in this study have not been isolated earlier from India. However, these serotypes have been isolated from diverse environmental sources from other parts of the world[17,18]. Using molecular techniques like RAPD, pulsed-field gel electrophoresis (PFGE) and 16S rRNA analysis, it would be of interest to know how the strains isolated from India differ from the well-known 'American' and 'European' strains[17]. All the *Y. enterocolitica* isolates obtained in this study were of biotype IA. Though generally regarded as non-pathogenic in the past, there has been a renewed interest in the pathogenicity of biotype 1A isolates. It has been shown that biotype 1A *Y. enterocolitica* may cause self-limiting enteritis without overt symptoms or even gastroenteritis similar to that caused by virulent biotypes[19]. In fact, in several parts of the world, significant number of *Y. enterocolitica* isolates from clinical cases of gastroenteritis belong to biotype 1A (ref. 20). It was suggested recently that isolates belonging to biotype 1A may be pathogenic by novel, as yet unknown mechanisms[21,22]. The pathogenic potential of the isolate obtained in this study is currently being investigated.

None of the earlier studies from India have reported isolation of *Y. intermedia*, which are characteristically rhamnose positive. Only *ca*. 3% of the total isolates are rhamnose negative and these constitute a rare biotype[23]. In this study, however, majority of the isolates was rhamnose negative and thus represented the rare biotype.

Y. enterocolitica or *Y. intermedia* could not be isolated from water samples collected from drains or the river Yamuna. This may possibly be due to dilution or the discharge of chemically-rich industrial effluents into the drains and the river, which might have adversely affected the survival of *Yersinia*[24]. However, this observation needs to be investigated further.

It has been reported that *Y. enterocolitica* from raw sewage and sewage sludge, where it can survive up to 1.5 years, can contaminate drinking water[25]. Since sewage effluents in India are discharged into surface waters

without any disinfection, it would be worthwhile to undertake further work on the prevalence of *Y. enterocolitica* in other types of environmental water.

1. Ostroff, S., *Contrib. Microbiol. Immunol.*, 1995, **13**, 5–10.
2. Abraham, M., Pai, M., Kang, G., Asokan, G. V., Mangesh, S. R., Bhattacharji, S. and Ramakrishna, B. S., *Indian J. Med. Res.*, 1997, **106**, 465–468.
3. Singh, G., Arora, N. K., Bhan, M. K., Ghai, O. P., Dhar, S. and Shriniwas, *Indian J. Pediatr.*, 1983, **50**, 39–42.
4. Varghese, A., Ramachandran, V. G. and Agarwal, D. S., *Indian J. Med. Res.*, 1984, **79**, 35–40.
5. Ram, S., Khurana, S., Singh, R., Sharma, S. and Vadehra, D. V., *ibid.*, 1987, **86**, 9–13.
6. Pramanik, A. K., Bhattacharyya, H. M., Chatterjee, A. and Sengupta, D. N., *Indian J. Anim. Health*, 1980, **19**, 79–81.
7. Verma, N. K. and Mishra, D. S., *Indian J. Anim. Sci.*, 1984, **54**, 659–662.
8. Singh, I. and Virdi, J. S., *Curr. Sci.*, 1999, **77**, 1019–1021.
9. Toora, S., Bala, A. S., Tiwari, R. P. and Singh, G., *Folia Microbiol.*, 1989, **34**, 151–156.
10. Krishnappa, G., Zaki, S. and Keshvamurthy, B. S., *Curr. Sci.*, 1980, **49**, 838–839.
11. Batra, H. V. and Garg, D. N., *Indian J. Anim. Sci.*, 1987, **57**, 639–646.
12. US Food and Drug Administration, in *Bacteriological Analytical Manual*, Washington DC, Association of Analytical Chemists, 1978, vol. XVIII, pp. 1–12.
13. Aulisio, C. C. G., Mehlman, I. J. and Sanders, A. C., *Appl. Environ. Microbiol.*, 1980, **39**, 135–140.
14. Schiemann, D. A., *Can. J. Microbiol.*, 1979, **25**, 1298–1304.
15. Barrow, G. I. and Feltham, R. K. A. (eds) *Cowan and Steel's Manual for Identification of Medical Bacteria*, Cambridge University Press, Cambridge, 1993, 3rd edn, pp. 94–164.
16. Ram, S., Khurana, S., Vadehra, D. V., Sharma, S. and Chinna, R. S., *Indian J. Med. Res.*, 1996, **103**, 253–258.
17. Robins-Browne, R. M., in *Food Microbiology: Fundamentals and Frontiers* (eds Doyle, M. P., Beuchat, L. R. and Montville, T. J.), American Society for Microbiology, Washington DC, 1997, pp. 192–215.
18. Bottone, E. J., *Microbes. Infect.*, 1999, **1**, 323–333.
19. Burnens, A. P., Frey, A. and Nicolet, J., *Epidemiol. Infect.*, 1996, **116**, 27–34.
20. Morris, Jr. J. G., Prado, V., Ferrecio, C., Robins-Browne, R. M., Bordun, A. M., Cayazzo, M., Kay, B. A. and Levine, M. M., *J. Clin. Microbiol.*, 1991, **29**, 2784–2788.
21. Grant, T., Bennett-Wood, V. and Robins-Browne, R. M., *Infect. Immun.*, 1998, **66**, 1113–1120.
22. *ibid.*, 1999, **67**, 4367–4375.
23. Brenner, D. J., Bercovier, H., Ursing, J., Alonso, J. M., Steigerwalt, A. G., Fanning, R. G., Carter, G. P. and Mollaret, H. H., *Curr. Microbiol.*, 1980, **4**, 207–212.
24. Marinelli, G., Innocenzo, C. D., Fabiani, L. and Leoni, V., *Appl. Environ. Microbiol.*, 1985, **49**, 1348–1349.
25. Langeland, G., *Acta Pathol. Microbiol. Immunol. Scand. Sect. B*, 1983, **91**, 179–185.

ACKNOWLEDGEMENTS. We thank Dr E. Carniel, Institut Pasteur, Paris, France and Dr B. Rowe, CPHL, Colindale, London for serotyping and biotyping of the strains.

Received 29 April 2000; revised accepted 9 June 2000

Semen characteristic profiles of men of different ages and duration of infertility

Kamala Gopalkrishnan†, Varsha Padwal, Donta Balaiah, P. Meherji, J. Gokral and R. Shah*

Department of Electron Microscopy, Institute for Research in Reproduction (ICMR), Parel, Mumbai 400 012, India
*Bhatia General Hospital, Mumbai 400 007, India

Semen characteristics were evaluated in men (n = 660) attending the infertility clinics, *vis-à-vis* age and duration of infertility. Men were divided into three groups according to age: 20–29 (n = 148), 30–39 (n = 437), > 40 (n = 75). Duration of infertility was grouped as follows: 1–5 years (n = 317), 6–10 years (n = 223), > 10 years (n = 120). The routine semen analysis was done according to WHO manual. Various semen parameters like semen volume, sperm count, motility, morphology and viability were evaluated. Values showed a decrease with increase in age and duration of infertility. It appears from the study that age and duration of infertility contributed to the decline in semen quality in men over the age of 40 years in the population studied. Thus these two parameters may possibly act as indicators to the quality of semen, specially in infertile men.

DATA on the reproductive function of men of different ages, specially of older men are rare. Regarding the reproductive and endocrine functions in ageing males, many questions still remain unanswered. Representative data in semen parameters are very few for ageing men. The study of Nieschlag *et al.*[1] have shown a significant decrease in sperm motility in older men along with ejaculate volume and fructose concentration. Only a small percentage of patients attending the infertility clinics are older than 40 years. It is well established that very few children are born to marriages in which husband is beyond the age of 60 years. This might be interpreted as an indication of reduced reproductive capacity with increasing age not only for the female partner but also for the male. Relationship between men's age and fecundity is difficult to approach directly due to many confounding factors like contraception, conception, abstinence, infection, degree of sexual activity, etc. It is well documented that in women with increasing age, there is a decrease in fertility[2]. An indirect but useful contribution regarding the relationship between age and fecundity as well as duration of infertility of the couple can be made by evaluating the influence of these on semen parameters. Thus retrospective analysis of the

†For correspondence. (e-mail: dirirr@vsnl.com)