

# Characteristics of *Yersinia pseudotuberculosis* isolated from animals in Brazil

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C.H.G. MARTINS, T.M. BAUAB AND D.P. FALCÃO. 1998. Strains (105) of *Yersinia pseudotuberculosis* isolated in Brazil between 1982 and 1990 were bio-serotyped. They were also studied for plasmid profile, autoagglutination and calcium dependence at 37 °C, Congo red uptake, pyrazinamidase activity, esculin hydrolysis, salicin fermentation and drug sensitivity: 95·24% were biotype 2, serogroup O:3; 2·86% were biotype 1, serogroup O:1; and 1·90% were biotype 2, non-agglutinable. Plasmids were found in 77·14% of the strains (one in each strain). There was total correlation between the presence of the virulence plasmid and autoagglutination, calcium dependence at 37 °C and Congo red uptake. The esculin, salicin and pyrazinamidase tests were not efficient in differentiating pathogenic from non-pathogenic *Y. pseudotuberculosis* isolates. All strains were highly sensitive to the drugs used. These results indicate that *Y. pseudotuberculosis* is a potential pathogen for humans in Brazil, especially because the bio-serogroups detected among animals are those most frequently associated with human diseases.

## INTRODUCTION

*Yersinia pseudotuberculosis* causes gastrointestinal infections associated with several clinical signs and symptoms in humans and animals, both in the form of outbreaks or isolated cases (Bisset 1979; Riet-Corrêa *et al.* 1990; Warth 1990; Butler 1994; Aleksic *et al.* 1995), and is classified in bioserogroups (Tsubokura and Aleksic 1995). The major mechanism of virulence of *Y. pseudotuberculosis*, as well as all other pathogenic *Yersinia* species, is invasiveness, which is mediated by genes *inv*, *ail* and *yadA*; the first two of these are chromosomal and the last, plasmidial. These genes are responsible for the production of the proteins Inv (invasin), Ail (attachment invasion locus) and YadA (*Yersinia* adhesin) (Paerregaard *et al.* 1990; Cornelis 1994; Carniel 1995). The virulence plasmid pYV (40–48 MDa) expresses different phenotypic characteristics such as autoagglutination at 37 °C, calcium dependence at 37 °C, and Congo red uptake (Cornelis 1994). A set of three tests has been proposed to separate pathogenic from non-pathogenic *Y. enterocolitica* strains: pyrazinamidase activity, esculin hydrolysis and salicin fermentation (Farmer III *et al.* 1992).

*colitica* strains: pyrazinamidase activity, esculin hydrolysis and salicin fermentation (Farmer III *et al.* 1992).

Studies on the behaviour of *Y. pseudotuberculosis* have demonstrated that the micro-organism is susceptible to a large number of antibiotics and chemotherapeutic agents (Falcão *et al.* 1978; Warth 1990; Butler 1994).

In Brazil, *Y. pseudotuberculosis* has been isolated only from animals. No human isolation has been described. The first report concerned its isolation from the liver of a rodent in 1979 (Hofer *et al.* 1979); this was later followed by further isolations from cattle, swine and buffalo (Falcão 1981; Oliveira *et al.* 1983; Suzumura 1984; Saridakis *et al.* 1988; Riet-Corrêa *et al.* 1990; Warth 1990).

The objective of the present study was to characterize further the *Y. pseudotuberculosis* strains isolated in Brazil, classify them in terms of origin, and submit them all together to biotyping and serotyping tests. Characteristics of virulence such as plasmid profile, autoagglutination at 37 °C, calcium dependence at 37 °C, and Congo red uptake were also studied. The strains were also tested for pyrazinamidase activity, esculin hydrolysis and salicin fermentation, and finally evaluated in terms of their response to drugs.

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## MATERIALS AND METHODS

### Bacterial strains

A total of 105 *Y. pseudotuberculosis* strains were studied. They were received at the *Yersinia* Reference Laboratory in Brazil, School of Pharmaceutical Sciences, Araraquara, UNESP, for complete identification, having been isolated between 1982 and 1990 in the States of Paraná and Rio Grande do Sul by different investigators (Barcellos and Castro 1981; Oliveira *et al.* 1983; Suzumura 1984; Saridakis *et al.* 1988; Riet-Corrêa *et al.* 1990; Warth 1990). They had been found in clinical materials from cattle, buffalo and swine, i.e. diarrhoeic faeces (57.14%), normal faeces (23.81%), material from the small intestine (8.57%), material from the large bowel (4.77%), mesenteric ganglia (2.86%), intestinal loops (1.90%) and others organs (0.95%). The general characteristics of the strains are presented in Table 1.

### Biotyping and serotyping

Determination of the four biovars and 14 serogroups of *Y. pseudotuberculosis* was carried out as described by Tsubokura and Aleksic (1995).

The biovars were determined by the biochemical reactions of raffinose, melibiose and Simmons citrate agar. The biochemical reactions were performed by conventional methods (Ewing 1986). Strains were inoculated, incubated at 25 °C, and the reactions read within 2–7 d. Biovar 1 was melibiose +, citrate and raffinose–; biovar 2 was melibiose, citrate and raffinose–; biovar 3 was melibiose and raffinose– and citrate +; biovar 4 was melibiose and raffinose +, citrate–.

The O antigens were determined by the slide agglutination test. The simplified antigenic scheme consisting of the 14 O serogroups was used. The reactions were considered positive

when complete agglutination of bacterial cells occurred within 1–2 min.

### Autoagglutination at 37 °C

Cultures were inoculated into two tubes of MR-VP (Methyl-Red-Voges-Proskauer) broth. One tube was incubated at 37 °C and the other at 25 °C. After 18–24 h, the tubes were observed for autoagglutination, taking care not to shake or disturb the sediment at the bottom and along the sides of the tube. Pathogenic strains containing the *Yersinia* virulence plasmid agglutinate at 37 °C but not at 25 °C. Strains lacking the virulence plasmid do not agglutinate at either temperature (Farmer III *et al.* 1992).

### Calcium dependence at 37 °C and Congo red uptake

These tests were performed on Congo red-magnesium Oxalate agar (CR-MOX) according to Riley and Toma (1989). The strains were first grown on blood agar plates at 25 °C for 24 h and then streaked onto CR-MOX agar medium, which allows visualization of calcium-dependent growth and uptake of Congo red dye on the same plate. The CR-MOX plates were incubated at 37 °C and observed for the presence of small red colonies at 24 and 48 h. Pathogenic strains contain the *Yersinia* virulence plasmid and are CR-MOX positive. Strains are CR-MOX negative if only large colourless colonies are present.

### Pyrazinamidase test

This test was performed as described by Kandolo and Wauters (1985). Strains were inoculated over the entire slant of pyrazinamidase agar (tryptic soy agar-yeast extract-pyrazine-

**Table 1** General characteristics of the 105 *Yersinia pseudotuberculosis* strains

Source/number of isolates	<i>Yersinia</i> strains	Place of isolation
Buffaloes: Diarrhoeic faeces, 5	Yp29; 80 to 83	Rio Grande do Sul
(34) Normal faeces, 25	Yp77 to 79; 84 to 105	Rio Grande do Sul
Organ, 1	Yp1	Paraná
Mesenteric ganglia, 3	Yp30 to 32	Rio Grande do Sul
Cattle: Diarrhoeic faeces, 54	Yp4 to 20; 22 to 25; 27, 28; 33, 34; 36 to 44; 47, 48; 50, 51; 53	Paraná
(70) to 55; 59; 61; 63 to 65; 67, 68; 70; 72 to 76		
Intestinal loop, 2	Yp2, 3	Paraná
Small intestine material, 9	Yp26; 35; 45; 57, 58; 60; 62 66; 69	Paraná
Large bowel material, 5	Yp46; 49; 52; 56; 71	Paraná
Swine: Diarrhoeic faeces, 1	Yp21	Paraná
(1)		

carboxamide-Tris-maleate buffer). Slants were inoculated with bacteria grown overnight on tryptic soy agar and incubated for 48 h at 25 °C. A 1 ml sample of a 1% (w/v) freshly prepared ferrous ammonium sulphate (aqueous) solution was then flooded on the slant. A reading was made after 15 min; a pink colour indicates the presence of pyrazinoic acid and is recorded as a pyrazinamidase-positive reaction. Negative cultures remained colourless.

### Salicin fermentation and esculin hydrolysis

Salicin fermentation and esculin hydrolysis were carried out according to Farmer III *et al.* (1992). Fermentation of salicin was determined in enteric fermentation base with 1% salicin and Andrade indicator. Esculin hydrolysis was determined in esculin broth. Both tests were performed by conventional methods (Ewing 1986). Strains were inoculated, incubated at 25 °C, and read at days 1, 2, 3 (or 4 or 5) and 7.

### Plasmid analysis

Isolation and extraction of plasmid DNA were done using the alkaline denaturation method of Birnboim and Doly (1979). The bacteria were inoculated into tryptic soy broth (TSB) and incubated at 25 °C for 24 h. Cultures were centrifuged, the pellets were resuspended in lysis solution and incubated in an ice bath; a mixture of NaCl and SDS was then added, the culture homogenized and again incubated in an ice bath. After the addition of sodium acetate solution and incubation in the ice bath, the vials were centrifuged. Supernatant fluids were recovered and plasmid DNA was precipitated with an equal volume of ethanol, followed by incubation at -20 °C for 18 h and centrifugation. Pellets were resuspended in Tris-acetate solution, 95% ethanol was added, and the mixture was homogenized and placed at -20 °C for 18 h. The precipitate was resuspended in TE buffer.

Plasmid DNA samples were electrophoresed through 0.7% agarose in TBE buffer (Meyers *et al.* 1976). The gels were stained with ethidium bromide solution, discoloured in water, and photographed.

Plasmid mass was determined with reference to the molecular weight plasmid standard, *Escherichia coli* 36R861 (110.0, 42.0, 23.9 and 4.6 MDa), included in all gels. The molecular weight, based on migration distances of samples and the plasmid standard included in each gel, was calculated using DNASTAR software (DNASTAR Computer Systems for Molecular Biology and Genetics, London, UK).

### Drugs resistance assays

The standard agar diffusion test was used according to Bauer *et al.* (1966). A few colonies were introduced into a tube containing trypticase soy broth. These tubes were incubated

overnight and the broth culture diluted to the density of the opacity standard (10- to 100-fold). For the sensitivity tests, Petri dishes were used with Mueller-Hinton agar. Plates were dried for about 30 min before inoculation and were used within 1 day of preparation. The bacterial broth suspension was streaked on the surface of the medium with a cotton swab. After the inoculum had dried (3-5 min) the disks were placed on the agar with flamed forceps and gently pressed down to ensure contact. The dishes were incubated for 24-48 h at 25 °C and the inhibition zones were recorded and interpreted according to the patterns. The following 13 antimicrobial agents were tested: chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline, gentamicin, nalidixic acid, erythromycin, polymyxin B, penicillin, ampicillin, cephalothin, amikacin, carbenicillin and tobramycin.

### RESULTS

The 105 *Y. pseudotuberculosis* strains were classified into biotypes 1 and 2 and serogroups O:1 and O:3, except for two strains which did not agglutinate with any of the sera available (NAG). The following distribution was observed: 100 isolates (95.24%) were biotype 2 serogroup O:3 (Yp 2/O:3); three (2.86%) were biotype 1 serogroup O:1 (Yp 1/O:1); and two (1.90%) were biotype 2 non-agglutinable (Yp 2/NAG).

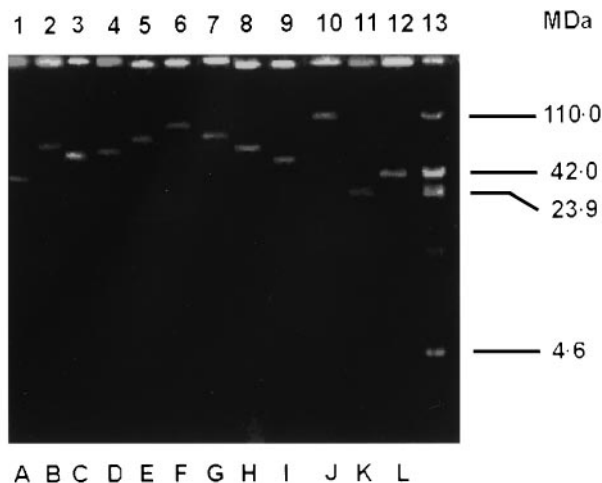
Plasmids were detected in 81 strains (77.14%) corresponding to 12 different plasmid profiles (A to L). Only one plasmid was found for each strain, although they had different molecular weights (Fig. 1). The results also showed that of the 81 strains with plasmids, 62 isolates (76.54%) were from sick animals and 19 (23.46%) from healthy animals. The 24 isolates (29.60%) without plasmids were also found in sick (18 isolates) and healthy (six isolates) animals. All strains with plasmids presented positive reactions for autoagglutination at 37 °C, calcium dependence at 37 °C and Congo red uptake.

The pyrazinamidase activity, esculin hydrolysis and salicin fermentation tests, which should be uniformly negative in pathogenic and positive in non-pathogenic isolates as is the case for *Y. enterocolitica*, presented anomalous results: 7.62% of the strains were positive for pyrazinamidase and 92.38% were negative; 97.14% were positive for esculin and 2.86% were negative; and 79.1% were positive for salicin and 20.9% were negative.

All strains were resistant to erythromycin. All were sensitive to ampicillin, carbenicillin, cephalothin, amikacin, gentamicin, tobramycin, nalidixic acid, sulfamethoxazole + trimethoprim, chloramphenicol, polymyxin B, and four were sensitive to tetracycline. All were moderately sensitive to penicillin and 101 to tetracycline.

### DISCUSSION

The fact that no *Y. pseudotuberculosis* strains isolated from humans have been sent to the *Yersinia* Reference Laboratory



**Fig. 1** Plasmid profiles of *Yersinia pseudotuberculosis* strains. Lane 1: profile A (40.5 MDa; Yp1). Lane 2: profile B (52.0 MDa; Yp21, 29, 31, 32, 57 to 59). Lane 3: profile C (47.0 MDa; Yp30, 84, 95, 96, 52 to 55, 60 to 63). Lane 4: profile D (51.0 MDa; Yp65 to 67, 69, 71 to 76). Lane 5: profile E (61.0 MDa; Yp68, 15 to 20, 81 to 83, 24). Lane 6: profile F (77.0 MDa; Yp79, 97, 99, 101 to 103, 105). Lane 7: profile G (64.0 MDa; Yp85, 86, 88, 90). Lane 8: profile H (55.0 MDa; Yp87, 89, 91 to 93, 23, 25 to 27, 37, 38). Lane 9: profile I (45.0 MDa; Yp40, 43, 44, 46, 48 to 51). Lane 10: profile J (110.0 MDa; Yp70). Lane 11: profile K (23.9 MDa; Yp10). Lane 12: profile L (42.1 MDa; Yp2, 3, 5, 6, 8, 9, 12, 13). Lane 13: plasmid standard *Escherichia coli* 36R861

seems to indicate that no human cases of yersiniosis pseudotuberculosis occur in Brazil. This is interesting as in other countries, such as Japan, the United States and Europe, the micro-organism has been isolated from humans (Bisset 1979; Tsubokura *et al.* 1989; Aleksic *et al.* 1995). The present data raise the question of whether yersiniosis pseudotuberculosis really does not occur in Brazil, or whether the disease(s) is being misdiagnosed, clinically or in the laboratory, because the serogroups detected in animals are those most frequently described as causing human disease in other countries (Inoue *et al.* 1991; Sato and Komazawa 1991; Aleksic *et al.* 1995). Also, the high predominance (95.24%) of the same bioserogroup (Yp 2/O:3) among affected animals appears to indicate a common origin of the isolated bacteria.

The three strains of *Y. pseudotuberculosis* Yp 1/O:1 with the virulence plasmid were isolated from sick buffaloes during three different outbreaks of diarrhoea that occurred from 1984 to 1987 in the state of Rio Grande do Sul (Riet-Corrêa *et al.* 1990). The two *Y. pseudotuberculosis* Yp 2/NAG strains, also with plasmids, were isolated from healthy buffaloes in 1990 in the state of Rio Grande do Sul (Falcão 1981) and probably belonged to serogroup O:3, having lost the antigen.

The biotyping of *Y. pseudotuberculosis* is quite recent and few reports are available. Tsubokura and Aleksic (1995) pro-

posed the four biotypes after studying *Y. pseudotuberculosis* strains isolated all over the world. Aleksic *et al.* (1995) tested 138 *Y. pseudotuberculosis* strains isolated in Germany over a period of 10 years and noticed the occurrence of only two biotypes (1 and 2) in strains of animal and human origin, with no correlation between biotype and serogroup. The present study shows a close correlation between biotype and serogroup.

The *Yersinia* virulence plasmid is defined as having a molecular weight of 40–48 MDa (Paerregaard *et al.* 1990; Cornelis 1994); in 53.1% of the strains in the present study, the molecular weight varied, as shown in Fig. 1. Other investigators have also observed variations in the molecular weight of the virulence plasmid (Shiozawa *et al.* 1987; Fantasia *et al.* 1991). An interesting result of the present study was the fact that strains with the plasmid and positive reaction for autoagglutination at 37 °C, calcium dependence at 37 °C and Congo red uptake were found in both sick and healthy animals. Therefore, the data were inconclusive when an attempt was made to correlate animal sickness or health with the presence of the virulence plasmid. The most likely explanation is that all isolates initially harboured the plasmid but, for the reason to be collection samples periodically struck for maintenance, some of them lost it.

As the pyrazinamidase, salicin and esculin tests did not present the uniform results shown in *Y. enterocolitica* (Farmer III *et al.* 1992), they were considered inefficient for *Y. pseudotuberculosis* and are therefore not recommended as tests of choice for discrimination between pathogenic and non-pathogenic strains.

The high percentage of drug sensitivity presented by *Y. pseudotuberculosis* strains, also reported by others (Falcão *et al.* 1978; Warth 1990; Butler 1994), seems to indicate genetic stability of the micro-organism in the presence of antimicrobial agents.

The present data lead to the conclusion that *Y. pseudotuberculosis*, although isolated only from animals, is a potential pathogen for humans in Brazil, especially considering that the serogroups detected in animals are those most frequently associated with human disease.

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