Comparison of antimicrobial susceptibility, β -lactamase production, plasmid analysis and serum bactericidal activity in *Edwardsiella tarda*, *E. ictaluri* and *E. hoshinae*

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Summary. Antimicrobial susceptibility profiles of clinical and environmental isolates of *Edwardsiella* demonstrate that the three species are susceptible to β -lactam antibiotics. All strains were susceptible to two quinolones tested and to gentamicin and doxycycline. *E. tarda* and *E. hoshinae* were resistant to clindamycin, whereas *E. ictaluri* was moderately susceptible. β -Lactamase was produced by all strains of *E. tarda*, but not by *E. hoshinae* or *E. ictaluri*. A 54-kb plasmid was detected in six of 13 *E. hoshinae* strains. Five of the 10 *E. tarda* isolates studied gave an identical plasmid pattern of four plasmids ranging in size from 76-kb to 5.0-kb. One strain exhibited a 54-kb plasmid; four strains did not contain plasmid DNA. All *E. ictaluri* isolates contained a 5.7-kb and a 4.9-kb plasmid. *E. tarda* and *E. ictaluri* strains were resistant to human serum 20%; 12 of 13 strains of *E. hoshinae* were also serum resistant. Serum resistance may play an important part in the pathogenicity of these species.

Introduction

Edwardsiella, a relatively unstudied genus in the family Enterobacteriaceae, is a motile, facultative anaerobic rod without the ability to ferment most carbohydrates.¹ Three species have been identified to date—*E. tarda*, *E. ictaluri* and *E. hoshinae*. The last species has only been recognised since 1980, after displaying the ability to ferment D-mannitol, D-trehalose and sucrose and salicin, in contrast to the other two species.² The three species have been isolated primarily from aquatic and cold-blooded animals; however, warm-blooded animals, including man, have been documented as harbouring the organisms.^{3–6}

E. tarda and *E. ictaluri* have been associated with infective disease. In man, *E. tarda* is an opportunist pathogen; most infections are acquired exogenously. *E. tarda* causes gastro-enteritis marked primarily by diarrhoea.^{4, 7–9} The diarrhoea may result from a heat-stable enterotoxin, although initial research indicates it to be more like the enterotoxin produced by *Salmonella* spp, which is of secondary pathogenic importance to the invasiveness of the bacterium.¹⁰ Cases of gastro-enteritis have been associated with nausea, vomiting, enteric fever and abdominal pain secondary to the infection.^{3, 7, 10–12} Extra-intestinal diseases are uncommon, but include hepatic abscesses, meningitis, wound infections, bacteraemia and osteomyelitis.^{8, 11–13}

The majority of pathogenicity studies, including antibiotic susceptibility studies and plasmid analysis, have been performed on *E. tarda*. This species is generally susceptible to those antimicrobial agents active against gram-negative bacteria, with the exception of colistin and polymyxin B.^{14–16} R plasmids conveying resistance to sulphonamide and tetracycline, or sulphonamide, tetracycline, streptomycin, kanamycin and chloramphenicol have been isolated from *E. tarda*,¹⁷ as well as a 123-kb plasmid of unknown content.¹⁸ A class D plasmid carrying tetracycline resistance alone, commonly found in marine and freshwater fish pathogens,¹⁹ has also been reported.

E. ictaluri, primarily associated with enteric septicemia of channel catfish,^{20, 21} is the leading cause of bacterial mortality in Mississippi delta catfish. Human pathogenicity has not been established to date.¹⁹ Two plasmids have been identified, 5.7-kb and 4.9-kb. Their significance has not been established,²⁰ but they are thought to harbour important virulence factors, or relate to metabolic processes. Its apparent lack of pathogenicity in man is likely to result from the relative absence of research on the species, or failure to recognise it in the clinical laboratory, as its biochemical profile is extremely close to that of *E. tarda*.

In 1980, *E. hoshinae* was established as a third species in the genus *Edwardsiella*. *E. hoshinae* isolates have been found in birds, reptiles and water.² Rarely, isolates from human faeces have been reported in patients without evidence of disease.¹ Because of its similarity to *E. tarda*, it might be expected to have a similar potential for disease in man. However, since

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little work has been done on the species, its pathogenic potential is unknown. At least one study has shown evidence for entero-invasiveness.²²

The potential exists for all three species of *Edwardsiella* to play a role in diarrhoeal disease, especially for individuals in wildlife or fish management and food processing occupations. Therefore, the specific aims of this study included: a comparative antibiotic susceptibility profile to determine antimicrobial agents that may be therapeutically useful; determination of β -lactamase production; assessment of the ability of the bacteria to survive serum bactericidal activity, a primary host defence against infection; and analysis of plasmids as potential carriers of antibiotic resistance and virulence factors. An investigation of these areas may better define the pathogenic potential of these bacteria, especially as *Edwardsiella* spp. and related bacteria are found in immunocompromised patients.^{23, 24}

Materials and methods

Bacterial strains

Ten strains each of *E. tarda* and *E. ictaluri* and 13 strains of *E. hoshinae* were studied. The strain designations and sources are listed in table I. Stock cultures were frozen at -76°C in Trypticase Soy Broth (TSB; Difco) with dimethyl sulphoxide (DMSO) 10%. Four of the isolates were duplicates, acquired from different laboratories: 9-66, 10-78, 2-78 (ATCC 33379) and 10-66 (ATCC 35051).

Identification methods

Routine microbiological testing procedures were used to confirm the species identification of the *E. hoshinae*, *E. tarda* and *E. ictaluri* strains. Grimont *et al.*² described criteria to differentiate *E. hoshinae* from similar species, including malonate utilisation and acid production from sucrose, D-trehalose and salicin. These criteria were followed in conjunction with the API 20E System (Analytab Products Inc., Plainview, New York) for each isolate. Morphological characteristics of *E. tarda* and *E. hoshinae* were obtained by growth on Trypticase Soy Agar (TSA; Difco) plates at 37°C incubated for 24 h and visualised by light microscopy. *E. ictaluri* isolates were grown at 25°C for 24–48 h.

The malonate utilisation test was performed by the modified Ewing malonate procedure.²⁵ Glucose 0.025% and yeast extract were added to stimulate initial growth of the organisms.

Antimicrobial susceptibility testing

The choice of antimicrobial agents was based on previously published susceptibility profiles of *E. tarda* and *E. ictaluri* and the potential efficacy of certain

antibiotics in gastro-intestinal infections.^{14, 16, 26, 27} Antimicrobial susceptibility test powders were obtained as follows: aztreonam (E. R. Squibb and Sons); ampicillin (Bristol Laboratories); piperacillin (Lederle Laboratories); imipenem (Merck, Sharp and Dohme); cefotaxime (Hoechst-Roussel Pharmaceuticals); ciprofloxacin (Miles); norfloxacin (Merck, Sharp and Dohme); clindamycin (UpJohn); gentamicin (Schering) and doxycycline (Pfizer).

A modification of the standard broth dilution method of the National Committee for Clinical Laboratory Standards²⁸ employing an inoculum of 5×10^3 cfu was used to determine minimum inhibitory (MIC) and bactericidal (MBC) concentrations. *E. ictaluri* isolates required incubation for 48 h at 25°C for visualisation of growth; this extended incubation period did not appear to affect adversely the result of susceptibility testing.

Serial two-fold broth dilutions were performed in Mueller Hinton Broth (MHB; Difco) for clindamycin, gentamicin and doxycycline. The range of concentrations was based on MICs previously reported for *Edwardsiella* and related species.^{15, 16} Two-fold broth dilutions for the remaining antibiotics, from 1.0 mg/L to 10 ng/L, were performed based on the results of preliminary 10-fold range-finding screenings (spot MICs). The MIC was defined as the lowest concentration of antibiotic at which no turbidity was observable; the MBC was the lowest concentration of the antibiotic from which a 99.9% reduction in the initial bacterial inoculum occurred.²⁹

β -lactamase testing

Four methods were used to detect β -lactamase: (1 and 2) chromogenic cephalosporin test as developed by O'Callaghan *et al.*,³⁰ utilising both whole cell suspensions (test 1) and cell suspensions,³¹ which had been sonicated for 5 min in 1-min bursts at 20 kc/s (45% intensity) (test 2); (3) the rapid acidometric method,^{32, 33} and (4) the Nitrocefin disk diffusion technique.³⁴ Cefinase^{TM} disks (BBL Microbiology Systems) were used according to the manufacturer's instructions.

Serum bactericidal activity

The bactericidal activity of serum against *E. tarda*, *E. ictaluri* and *E. hoshinae* was measured by the method of Wiemer *et al.*³⁵ Fresh whole blood was obtained with consent from healthy volunteers from the University of Illinois College of Medicine at Peoria. Pooled serum was frozen at -70°C in 2-ml volumes and thawed immediately before use. Heat inactivated serum was used as a growth control. A strain was considered to be serum sensitive if there was at least a 10-fold decrease in viable counts between time zero and 120 min as compared to the control; resistant strains demonstrated growth, or no change in counts from the original inoculum.³⁶

Table I. Source of *Edwardsiella* strains

Species	Strain no.	Specimen	Source
<i>E. tarda</i>	11	NA	1
	16	Stool	1
	3276	Stool	1
	1079	Stool	1
	11277	Stool	1
	51778	Stool	1
	1228417	Stool	1
	1230008	Stool	1
	HVA643	Stool	1
	HVA986	Sputum	1
	AL-5	Channel catfish	2
	1354	Channel catfish	2
	6012	Channel catfish	3
<i>E. ictaluri</i>	6013	Channel catfish	3
	33202	Channel catfish	2
	S89-514	Channel catfish-kidney	4
	S89-520	Channel catfish-kidney	4
	S89-521	Channel catfish-kidney	4
	S87-661	Channel catfish-kidney	4
	S87-637	Channel catfish-kidney	4
<i>E. hoshinae</i>	1-78	Male puffin	1
	EH-1	NA	1
	9-66 (dry)	Monitor lizard	1
	ATCC 33379	Female puffin	1
	ATCC 35051	NA	1
	2-78	Female puffin	5
	2-66	Monitor lizard	5
	9-66	Monitor lizard	5
	10-66	Monitor lizard	5
	12-67	Lizard	5
	1-78	Male puffin	5
	3-78	Flamingo	5
	420-72	Water	5

NA, not available.

1, J. M. Janda, California State Department of Health Service, Berkeley, CA; 2, C. Lobb, University of Mississippi; 3, E. Shotts, University of Georgia (courtesy of J. M. Janda); 4, T. Santucci Mississippi Cooperative Service, Stoneville, MS (courtesy of S. Hayasaka, Clemson University); 5, P. A. D. Grimont, Pasteur Institute, Paris.

Plasmid screening

Two methods were used to extract plasmid DNA. Strains were initially screened for plasmid DNA by the methods of Kado and Liu³⁷ and that of Portnoy and White.³⁸⁻⁴⁰

Agarose gel electrophoresis was performed according to the method of Meyers *et al.*,⁴¹ with 15- μ l samples separated through agarose 0.7% gel.

Approximate plasmid size was determined by interpolation of plots of the log of the mol. wt *versus* relative mobility of reference strains harbouring known plasmids of both low and intermediate mol. wt ranging from 1.9-kb to 54-kb.⁴²

Light and electronmicroscopy

Light microscopy (American Optical 150 microscope) was used to examine isolates for antibiotic-induced morphological alterations.⁴³ Ten 1000 $\times$ oil-immersion fields were examined for each strain, at each of 10 antibiotic concentrations. Elongation and filamentation were defined according to Lorian:⁴⁴ a bacillus was considered to be elongated when the cell

length had at least doubled. A rod which was > 10 μ m in length was considered to be a filament.

Imipenem was used to evaluate morphological defects by transmission electronmicroscopy. Cell suspension of strain EH-1 in concentrations of imipenem from 7.8–156 μ g/L (one and two tubes below the MIC, respectively) were used as a representative isolate. Standard electronmicroscopy procedures for embedding dehydrating, sectioning and staining were employed.^{45,46}

Results

Identification

All colonies grown on TSA were flat, small (0.5–2 mm in diameter) and clear with smooth borders. However, *E. hoshinae* strain 9-66 from Janda exhibited a colony that was initially a more dry and friable consistency compared to strain 9-66 obtained from Grimont. The results of biochemical characterisation of the isolates with the API 20E system and conventional biochemical tests are summarised in table II. *E. ictaluri* isolates were incubated for 48 h at 25°C and 37°C. Citrate was utilised by *E. ictaluri* isolates at 25°C but not at 37°C. Lysine decarboxylase activity was variable; all other biochemical tests gave equivocal results at both temperatures.

The ability of *E. hoshinae* to utilise sodium malonate as a sole carbon source is a major character differentiating this species from *E. tarda* and *E. ictaluri*.² All *E. hoshinae* isolates were malonate positive; *E. tarda* and *E. ictaluri* isolates were malonate negative.

Antimicrobial susceptibility testing and morphological defects

All the *Edwardsiella* isolates were susceptible to the five β -lactams tested (MIC $\leq$ 0.31 mg/L), with greatest sensitivity to cefotaxime and aztreonam (MICs < 0.01 mg/L) (table III). Imipenem had the highest MIC values of any β -lactam antibiotic tested against *E. ictaluri*, but when achievable blood levels were taken into account this was not significant.

The three *Edwardsiella* spp. were exquisitely susceptible to the quinolones. Ciprofloxacin had at least a 100-fold greater activity against *E. tarda* and *E. ictaluri* than against *E. hoshinae*. Gentamicin and doxycycline MICs were $\leq$ 0.625 mg/L. Clindamycin resistance was demonstrated in *E. tarda* and *E. hoshinae*; *E. ictaluri* was moderately susceptible (table III).

The percentage of MICs equalling MBCs are summarised in table IV. Marked variability was noted among the strains when comparing MICs *versus* MBCs for the β -lactam antibiotics. With the exception of *E. ictaluri* and ciprofloxacin, 90% of the MBCs equalled MICs for the quinolones. The inhibitors of protein synthesis also exhibited strain variability when comparing MICs to MBCs; none of the strains of *E. tarda* or *E. ictaluri* had MICs equal to MBCs for clinda-

Table II. Biochemical characteristics of *Edwardsiella* isolates

Characteristic	Results obtained with			
	<i>E. tarda</i>	<i>E. ictaluri</i>		<i>E. hoshinae</i>
		25°C	37°C	
API20E				
ONPG	—	—	—	—
Arginine dihydrolase	—	—	—	—
Lysine decarboxylase	+	+	+	+
Ornithine decarboxylase	+	—	—	+
Citrate	+	+	—	+
H ₂ S	+	—	—	+
Urea	—	—	—	—
Tryptophane deaminase	—	—	—	—
Indole	+	—	—	+
Voges-Proskauer (VP)	—	—	—	—
Gelatin liquefaction	—	—	—	—
Glucose	+	+	+	+
Mannitol	—	—	—	+
Inositol	—	—	—	—
Sorbitol	—	—	—	—
Rhamnose	—	—	—	—
Sucrose	—	—	—	+
Melibiose	—	—	—	—
Amygdalin	—	—	—	—
(L +) Arabinose	—	—	—	—
Conventional biochemical tests				
Malonate	—	—	—	+
β -Lactamase	+	—	—	—

* Strains 514 and 637 gave negative results.

† Strains AL5, 514 and 521 gave negative results.

‡ Strain 2-66 gave a negative result.

§ Strains 490-72 and 9-66 gave negative results.

Table III. MIC₅₀ and MIC₉₀ for *Edwardsiella* species of a range of antibiotics

Antibiotic	<i>E. tarda</i>		<i>E. ictaluri</i>		<i>E. hoshinae</i>	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
<i>β-lactam agents</i>						
Ampicillin	0.1*	0.1	0.156	0.156	0.1	0.1
Piperacillin	0.1	0.1	0.156	0.156	0.1	0.1
Imipenem	0.156	0.156	0.31	0.31	0.1	0.1
Aztreonam	0.01	0.012	0.01	0.01	0.1	0.1
Cefotaxime	0.01	0.01	0.01	0.01	0.0001	0.001
<i>Quinolones</i>						
Ciprofloxacin	0.0001*	0.0001	0.0001	0.0001	0.01	0.01
Norfloxacin	0.01	0.01	0.01	0.01	0.01	0.01
<i>Protein synthesis inhibitors</i>						
Clindamycin	6.25	12.5	6.25	6.25	12.5	25.0
Gentamicin	0.625	0.625	0.31	0.31	0.078	0.156
Doxycycline	0.31	0.31	0.078	0.156	0.078	0.156

* mg/L.

mycin. However, compared to doxycycline and clindamycin, gentamicin exhibited the greatest bactericidal activity.

Characteristic β -lactam-induced morphological defects were observed: cell elongation at lower concentrations of antibiotic followed by gradual increase to mid-cell defects and spheroplasts as concentrations approached the MIC. Imipenem, with specific affinity for penicillin-binding protein 2, caused formation of spheroplast structures at all concentrations, as expected (fig. 1A and B, fig. 2).

β -lactamase production

Irrespective of the method used, none of the *E. ictaluri* or *E. hoshinae* isolates produced β -lactamase in quantities detectable by our procedures. In contrast, all 10 strains of *E. tarda* exhibited β -lactamase activity with all methods.

Serum bactericidal activity

All strains of *E. tarda* and *E. ictaluri* were resistant to 20% serum; 12 of the 13 strains of *E. hoshinae* were

Table IV. Relationship between MIC and MBC.

Antibiotic	Percentage of MBCs equalling MICs for		
	<i>E. tarda</i>	<i>E. ictaluri</i>	<i>E. hoshinae</i>
Ampicillin	10	20	77
Piperacillin	20	50	77
Imipenem	50	60	62
Aztreonam	80	60	85
Cefotaxime	100	50	77
Ciprofloxacin	90	50	90
Norfloxacin	90	90	90
Clindamycin	0	0	10
Gentamicin	40	70	30
Doxycycline	0	40	8

resistant (fig. 3). *E. hoshinae* strain 35051 was susceptible to the pooled human serum, with a 10-fold decrease occurring after 120 min (fig. 4). Complete bactericidal activity did not occur, as viable organisms were recovered after 120 min. Although *E. hoshinae* strains 35051 and 10-66 are the same but obtained from different laboratories, only strain 35051 exhibited serum susceptibility.

Plasmid screening

Although both the Kado and Liu, and the Portnoy and White methods of plasmid extraction were successful in the identification of plasmid DNA from our strains, the Portnoy method yielded clearer resolution of the individual plasmid DNA bands with less contamination by extraneous proteins (fig. 5).

Four plasmid bands were detected in five of the 10 *E. tarda* isolates screened (76-kb, 7.6-kb, 6.5-kb and 5.0-kb). One isolate exhibited a single 54-kb band; four *E. tarda* strains lacked plasmid DNA. All the *E. ictaluri* isolates contained a 5.7-kb and a 4.9-kb plasmid. Of the *E. hoshinae* isolates, six (46%) of the 13 strains screened contained one plasmid of c. 54-kb. The remaining seven strains did not contain detectable plasmid DNA.

Discussion

Although the three species of *Edwardsiella* have been shown to be related by DNA-DNA hybridisation,¹ there is no evidence to indicate that *E. hoshinae* or *E. ictaluri* has the same pathogenic potential as *E. tarda*. In this study, a collection of known strains of *Edwardsiella* spp. was examined by several procedures to assess pathogenic potential.

The antibiotics chosen for this study included those known to be effective in controlling bacteria capable of gastro-intestinal infection, most notably similar gram-negative pathogens, from our previous studies.⁴⁷ Susceptibility profiles for *E. hoshinae* were similar to those of *E. tarda* and *E. ictaluri* for the β -lactam agents, quinolones and the protein synthesis inhibitors gentamicin and doxycycline. All showed marked susceptibility, especially to the quinolones. Both *E. hoshinae* and *E. tarda* were resistant to clindamycin (MIC $\geq$ 25 mg/L); *E. ictaluri* exhibited moderate susceptibility.

Susceptibility to the β -lactams included the penicillins, a monobactam, a carbapenem and a third generation cephalosporin. The absence of β -lactamase

Fig. 1. Spheroplasts of *E. hoshinae* EH-1 induced by imipenem, which are at least twice the normal width of bacterial cells. The Tri-laminar outer wall structure has been lost due to antibiotic exposure (►) (A, $\times 40000$; B, $\times 52000$).

Fig. 2. Shedding of lipopolysaccharide from a spheroplast of *E. hoshinae* EH-1 (►) after exposure to imipenem. This process leads to loss of the tri-laminar cell wall structure ($\times 65000$).

Fig. 3. Composite serum bactericidal susceptibility curves of 10 *E. tarda* isolates illustrating resistance to pooled human serum by the absence of a 10-fold decrease in viable counts after 120 min. PHS, 20% active serum (—+—); HIS, 20% inactivated serum (—Δ—).

in *E. hoshinae* and *E. ictaluri* may be of little importance because of the limited distribution of these species in the environment.

In contrast, *E. tarda*, a known human pathogen, produces β -lactamase⁴⁷ and has wider environmental distribution. Reinhardt *et al.*¹⁵ also noted β -lactamase production by 29 *E. tarda* strains that exhibited low MICs for β -lactam and antibiotics as did Clarke *et al.*⁴⁸ in a recent study of 22 *E. tarda* isolates. The production of β -lactamase by *E. tarda* isolates emphasises the fact that prediction of susceptibility to β -lactam antibiotics may not be reliable. Enzyme concentration, the hydro-

lytic efficiency of the enzyme, binding affinity and permeability of the outer membrane must also be considered as reasons for lack of β -lactam resistance when β -lactamase is detected.⁴⁹

Five of the 10 *E. tarda* isolates contained four plasmid bands ranging from 76-kb to 5.0-kb. Six of the 13 strains of *E. hoshinae* and one strain of *E. tarda* harboured a 54-kb plasmid of unknown function; all *E. ictaluri* isolates screened contained 5.7-kb and 4.9-kb plasmids, confirming the findings of Lobb and Rhoades²⁰ and Reid and Boyle.⁵⁰ No distinct pattern arose to indicate that clindamycin resistance correlated

Fig. 4. Serum bactericidal susceptibility curve illustrating susceptibility of *E. hoshinae* 35051 to pooled human serum by the presence of a 10-fold decrease in viable counts after 120 min. PHS, 20% active serum (—+—); HIS, 20% inactivated serum (---Δ---).

Fig. 5. Agarose gel electrophoresis of DNA lysates of *Edwardsiella* isolates and *Escherichia coli*: 15 μ l of lysate was electrophoresed at a constant voltage of 60V in agarose 0.7%. Lane A, *Esch. coli* J53(sa); B, *Esch. coli* J53(RPH); C, *Esch. coli* V517(PVA 517); D, *E. tarda* 51778; E, *E. ictaluri* 6012; F, *E. hoshinae* (EH-1).

with the presence of any of the plasmids. It is possible that testing a more extensive antibiotic profile could elucidate plasmid function in these isolates.

With one exception, all *Edwardsiella* spp. tested were resistant to the bactericidal effects of 20% human serum. Resistance to the killing effects of serum has been shown to correlate with the ability of bacteria to invade and survive in the bloodstream.³⁶ The mechanism of action for the findings are unknown. Serum resistance may be mediated by the failure of complement components to integrate into the cell membrane because of the presence of repelling lipopolysaccharides or outer-membrane proteins. Some bacterial species have been found to have the ability to bind serum proteins (albumin, fibrinogen, IgG) to their surfaces and thus alter the host's ability to destroy them by serum-mediated mechanisms.⁵¹ Plasmid profiles did not correlate with serum susceptibility or resistance.

During serum bactericidal testing, *E. hoshinae* isolates exhibited considerably higher colony counts than expected in spite of apparent appropriate dilution as determined by spectrophotometry. This phenomenon may be secondary to cell clumping or agglutination.⁵² Bergan *et al.*²⁶ have proposed that agglutination may be a useful indicator for virulence. Auto-agglutination (AA phenotype) of mesophilic aeromonads was found to be associated with higher levels of pathogenicity in mice. Therefore, auto-agglutination could be a plausible explanation for both the noted dilution phenomenon as well as the serum resistance, since auto-agglutination provides a mechanism for resistance to complement-mediated lysis.^{53, 54}

Serum-resistant *Edwardsiella* spp. possess the ability to avoid the bactericidal effects of serum, which

provides evidence that they could survive *in vivo* and to cause disease in man. Further studies may be warranted, including studies at higher serum concentrations (e.g. 60%). As *Edwardsiella* sp. are found increasingly in association with immunocompromised or

severely ill patients, serum resistance may provide a mechanism of action for *Edwardsiella* spp. to cause disease. Fortunately, as this investigation has shown, these species of bacteria are susceptible to antibiotics commonly used in medical practice.

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