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Virulence Factors of *Haemophilus ducreyi*

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We investigated the susceptibility of virulent and avirulent strains of *Haemophilus ducreyi* to the bactericidal activity of normal human serum and to phagocytosis and killing by human polymorphonuclear leukocytes (PMNL). Strains were defined as virulent if intradermal inoculation into a rabbit produced a typical necrotic lesion. Nonvirulent strains produced no cutaneous lesions in rabbits. Virulent strains were resistant to the complement-mediated lethal action of normal human and rabbit sera, whereas avirulent strains were susceptible (>95% kill, 60 min). Virulent strains were relatively resistant to phagocytosis and killing by human PMNL, in contrast to the avirulent strains. In past studies polymyxin resistance has been correlated with virulence in *H. ducreyi*. In our studies, polymyxin resistance could not be correlated with virulence, since polymyxin-sensitive mutants obtained from polymyxin-resistant parent strains remained virulent for rabbits and resistant to bactericidal action of normal serum and phagocytosis and killing by human PMNL. Similarly, polymyxin-resistant mutants obtained from polymyxin-sensitive parent strains remained avirulent for rabbits and susceptible to bactericidal action of normal serum and PMNL. The acquisition of polymyxin resistance was accompanied by the loss of a 47,000-molecular-weight protein. The association of serum resistance and resistance to phagocytosis and killing by human PMNL with virulent strains, as defined by the rabbit intradermal test, suggests that these factors may mediate the pathogenicity of *H. ducreyi*.

Haemophilus ducreyi is the etiological agent of chancroid, a sexually transmitted disease. The virulence of this organism has been studied by a number of investigators (4, 5, 7, 22). The only accepted test of virulence has been the rabbit intradermal test (4, 5), which results in necrosis and eschar formation. Dienst (4) demonstrated that virulent strains produced a localized skin lesion in rabbits and humans after intracutaneous inoculations with 48-h-old cultures, whereas avirulent strains did not produce localized skin lesions. Recently, isolated strains lose their virulence after repeated passages on artificial media. Virulence has been related to antibiotic resistance (18, 22), especially polymyxin B (7). Virulent strains positive in the rabbit intradermal test were resistant to the bactericidal action of polymyxin B (minimal inhibitory concentration, ≥32 μg/ml), whereas the avirulent strains were susceptible. This study examined the susceptibility of *H. ducreyi* strains to the bactericidal action of normal human serum (NHS) and normal rabbit serum and to phagocytosis and killing by human polymorphonuclear leukocytes (PMNL). These characteristics have been shown to be important host defense determinants for other gram-negative bacteria (1, 6, 12, 17). These parameters for *H. ducreyi* were compared with the rabbit intradermal test and with polymyxin resistance as virulence markers.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. ducreyi* strains A75, A76, and A77 are reference strains obtained from the Pasteur Institute in Paris; strains 409, O78, and C148 are isolates obtained from Kenya; strain 35000 is a Winnipeg isolate; and strain C148^r is a rifampin-resistant isogenic strain of strain C148. All strains except C148^r were originally isolated from ulcers of patients with clinical chancroid.

Strains were kept as frozen skimmed milk stock cultures at −70°C. When needed, strains were cultured on hemoglobin agar composed of gonococcal medium base and CVA enrichment (GIBCO Diagnostics, Madison, Wis.) and incubated at 35°C under 5% CO_2 and high humidity.

Growth on hemoglobin agar surfaces was identified as *H. ducreyi* by the nonmucoid, yellow-gray appearance and the cohesive nature of the colonies, which enabled them to be pushed intact across the agar surface. All strains required hemin (X factor) for growth. Biochemically, they were oxidase positive and catalase, urease, and ornithine decarboxylase negative, did not produce indole or H_2S, and did not ferment glucose, sucrose, lactose, mannitol, or xylose. Microscopically, all organisms were small, pleomorphic, gram-negative rods.

Virulence testing. Virulence of stock strains was tested by the intradermal injection (4, 5) of 0.20 ml of a sheep brain heart infusion broth suspension containing 10^9 CFU of *H. ducreyi* per ml into 2- to 3-kg, 1-year-old female rabbits. Induration and necrosis were determined daily from day 1 until day 11 postinoculation. The criteria for virulence were as follows: by day 4, induration exceeded 0.5 cm and the lesion progressed to an eschar by day 11.

Adaptive resistance to polymyxin. Strains inhibited by ≤32 μg of polymyxin B per ml were considered susceptible. Polymyxin-susceptible strains were made resistant by cultivating the strains on hemoglobin agar with increasing concentrations of polymyxin, until they grew on media containing 250 μg of polymyxin per ml.

Polymyxin-sensitive mutants. Polymyxin-sensitive mutants of *H. ducreyi* were obtained from polymyxin-resistant parent strains by treatment with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. An overnight culture (18 h) on hemoglobin agar was scraped into sheep brain heart infusion broth supplemented with hemin (50 μg/ml), glucose (50 mg/ml), glutamine (5 mg/ml), and cysteine (12.5 mg/ml). A 0.5-ml portion of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.5 μg/ml) was added to 2 ml of bacterial suspension (approximately 10^9

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CFU/ml) along with 2 ml of heated fetal calf serum. After 2 h of incubation at 35°C, the cell suspension was centrifuged at 10,000 × *g* for 5 min, the pellet was washed twice in fresh broth, and appropriate dilutions were plated on hemoglobin agar supplemented with CVA enrichment. The survivors were screened for loss of resistance to polymyxin by the replica plating method (9).

Serum bactericidal assay. Blood from five healthy young adults with no history of chancroid was allowed to clot at room temperature for 30 min and then centrifuged at 2,000 × *g* for 10 min at 4°C. The serum specimens were pooled and stored in small samples at −70°C until required. Normal rabbits were bled from the ear, and serum was separated and stored similarly. A 16-h culture of *H. ducreyi* was harvested into proteose peptone-saline (PPS) buffer (1% Difco Proteose Peptone in physiological saline), pH 7.2, and washed twice. The cell suspension was diluted to a concentration of 5×10^8 CFU/ml with PPS. At time zero, 0.1 ml of the bacterial suspension (5×10^7 CFU) was added to a tube containing 0.4 ml of PPS and 0.5 ml of undiluted normal serum. Control tubes contained heat-inactivated normal serum. Tubes were incubated at 35°C and rotated on a rotator at 10 rpm. At time intervals of 0, 30, 60, and 120 min, samples were taken and serially diluted in PPS and 0.3 ml of the appropriate 10-fold dilutions was plated onto hemoglobin agar in 6, 0.05-ml drops for the determination of viable counts by the Miles-Misra method (10). Killing was measured by comparing the survival of *H. ducreyi* in normal and heat-inactivated (56°C for 30 min) serum. Serum resistance was defined as a <50% reduction of the original bacterial inoculum during 120 min of incubation in 50% serum.

PMNL isolation. Human PMNL were prepared as described by Quie et al. (15), with slight modifications. Heparinized venous blood from a single donor with no history of chancroid was sedimented for 60 min by adding 3 ml of 6% dextran in saline to 10 ml of blood. The supernatant fluid containing leukocytes and a few erythrocytes was collected, and the cells were recovered by centrifugation and washed twice in saline. The pellet was suspended in 10 ml of 0.84% NH_4Cl and incubated in a water bath at 37°C for 10 min to lyse the residual erythrocytes, after which the cells were washed free of the NH_4Cl with Hanks balanced salt solution (HBSS) containing 0.1% gelatin (pH 7.2). The viability of the cells was determined by trypan blue exclusion, and the cells were counted in a hemocytometer and resuspended in HBSS to give a concentration of 10^7 PMNL/ml.

Phagocytosis assays. The susceptibility of *H. ducreyi* strains to the killing activity of human PMNL was determined, using the method described by Quie et al. (15) with some modifications. The reaction mixture in each siliconized tube (12 by 75 cm) consisted of 5×10^7 CFU (0.1 ml), 5×10^6 human PMNL (0.5 ml), 2% NHS, and HBSS (containing 0.1% gelatin) in a final volume of 1 ml. The control tubes contained no PMNL. In some experiments heat-inactivated serum was used. The tubes were rotated on a rotator at 10 rpm. Samples were taken at zero time and after 30, 60, and 120 min of incubation. These were serially diluted in PPS and vortexed to lyse PMNL, and dilutions were plated on hemoglobin agar. Colonies were counted at 35°C to determine the total number of surviving bacteria. Killing was expressed as the percentage of the number of viable bacteria recovered from samples with no incubation time, i.e., percentage killed = [viable count (t_0) − viable count (t)]/[viable count (t_0)] × 100.

The kinetics of ingestion of *H. ducreyi* by human PMNL was also determined with a sensitive acridine orange fluorescence method described by Smith and Rommel (19), combined with the use of crystal violet to quench fluorescence of noningested organisms (8). Acridine orange-labeled bacteria fluoresce green when viable and red if nonviable (19). Leukocyte suspensions (0.2 ml) obtained by the dextran sedimentation method were deposited on a series of no. 1 cover slips (22 by 22 mm) and incubated at 35°C in a humidified 5% CO_2 incubator for 60 min. The cover slips with monolayers of leukocytes were rinsed with HBSS (at 35°C, pH 7.2). Bacterial cells (10^8 CFU/ml of HBSS plus 0.1% gelatin) were preopsonized in 2% NHS for 15 min at 35°C, and 0.2 ml of the preopsonized bacterial cells was added to each cover slip. The cover slips were reincubated at 35°C in a humidified 5% CO_2 incubator. Cover slips were taken out at various times and washed in HBSS at 35°C. The adherent cells were stained with acridine orange (14.4 mg/liter) in Gey balanced salt solution (pH 7.2) at 35°C for 45 s and washed in HBSS at 35°C to remove excess stain. To distinguish intracellular organisms, the adherent cells were flooded with a crystal violet solution (1 mg/ml in 0.15 M NaCl) at 35°C for 45 s. The cover slips were washed in HBSS, pH 7.2, to remove excess dye, and each cover slip was placed on a glass slide with the adherent leukocytes in contact with the slide. The wet-mount preparation was examined under a Leitz UV epiluminescent microscope, using a Leitz ×100 oil immersion objective. Two hundred cells were counted, and the percentage of the leukocytes containing ingested *H. ducreyi* cell(s) was referred to as percent phagocytosis. This estimate did not take into account the viability of ingested cells.

Analysis of outer membrane proteins. Sarcosinate-insoluble outer membrane proteins from a 24-h culture of *H. ducreyi* grown on hemoglobin agar were prepared according to the procedure described by Barenkamp et al. (2). The

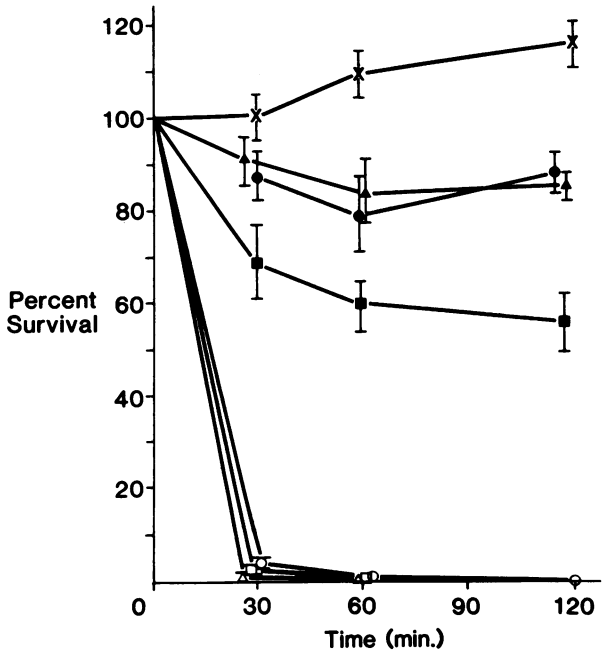

FIG. 1. Bactericidal effect of 50% pooled NHS on *H. ducreyi* strains 35000 (●), 409 (▲), C148 (■), A77 (○), A75 (△), and A76 (□). Heat treatment abolished bactericidal activity of NHS (data shown only for *H. ducreyi* A76 [×]). Data points represent the mean ± standard deviations of three separate experiments.

protein preparations were solubilized by boiling for 5 min in 2% sodium dodecyl sulfate–10% glycerol–5% 2-mercaptoethanol–0.0625 M Tris-hydrochloride (pH 6.8). Samples containing 30 μg of proteins were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 1.5-mm-thick slab gel, as previously described (20). The protein content of the samples was measured by the Bradford method (3).

RESULTS

Survival of *H. ducreyi* in various suspending fluids (pH 7.2). The results of a preliminary experiment on the survival of *H. ducreyi* strains indicate that PPS (pH 7.2) or PPS supplemented with 2% NHS supports the viability of the strains for 3 h of incubation at 35°C (data not shown). HBSS supplemented with 0.1% gelatin and 2% NHS also supports the viability of the strains for 3 h of incubation at 35°C, whereas HBSS supplemented with 0.1% gelatin fails to sustain viability. Strains die quickly in saline and phosphate-buffered saline (0% survival within 60 min). PPS was therefore used in all subsequent experiments.

Sensitivity to bactericidal action of NHS. Virulent strains of *H. ducreyi* 409, C148, and 35000 as determined by the rabbit intradermal test were resistant to the bactericidal action of NHS (<50% kill in 120 min). Avirulent strains A75, A77, and A76 were extremely sensitive (Fig. 1), with no survivors after 120 min of incubation in 50% NHS. Serum concentrations above 5% were bactericidal for these serum-sensitive strains, whereas the survival of the serum-resistant strains improved in fluids with <50% NHS (data not shown). All strains were unaffected by heat-inactivated serum.

Results obtained for these strains in normal rabbit serum were similar to those with NHS.

Phagocytosis of *H. ducreyi* by human PMNL. Two methods of assessing phagocytosis have been used. The first required lysis of the PMNL and determination of viable counts. Results shown in Fig. 2 indicate that virulent strains 409, 35000, and C148 were more resistant to killing than avirulent strains A77, A75, and A76. Strains were less susceptible to the killing activity of human PMNL when incubated with heat-inactivated serum.

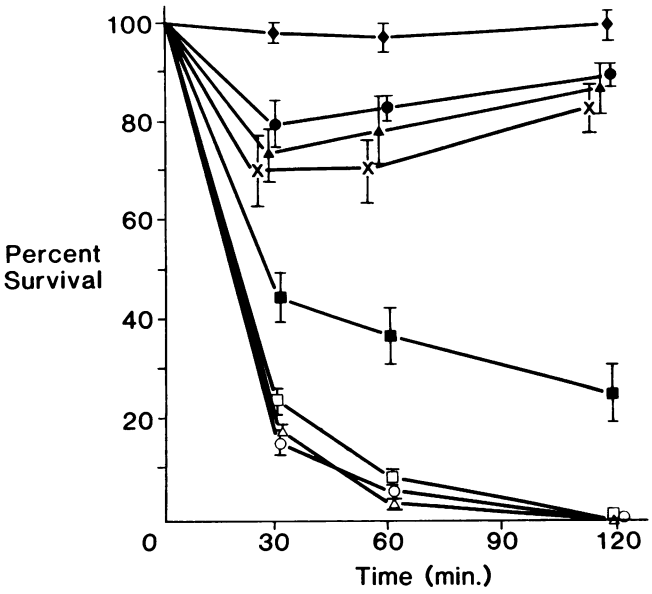

FIG. 2. Susceptibility of *H. ducreyi* strains to bactericidal action of human PMNL: *H. ducreyi* strains 409 (●), 35000 (▲), C148 (×), A77 (□), A75 (△), and A76 (○), incubated with PMNL + 2% NHS. *H. ducreyi* strain A77 was incubated with PMNL + 2% heated NHS (■). Control: *H. ducreyi* incubated with 2% NHS only (data shown for *H. ducreyi* A77 [◆].) Data points represent the mean ± standard deviation of three separate experiments.

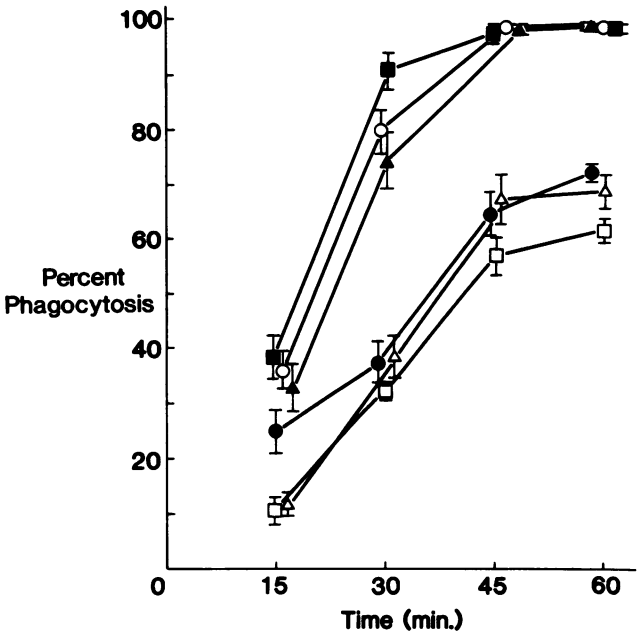

FIG. 3. Kinetics of phagocytosis of *H. ducreyi* strains by human PMNL: *H. ducreyi* strains 409 (□), C148 (●), 35000 (△), A77 (○), A75 (■), and A76 (▲). Data points represent the mean ± standard deviation of three separate experiments.

The second method of phagocytosis assay required the use of acridine orange and crystal violet, the latter quenching fluorescence of organisms not actually ingested by the PMNL. In Fig. 3, it is observed that a significantly higher percentage of avirulent strains was ingested in relation to

TABLE 1. Relationship between polymyxin resistance and virulence of *H. ducreyi* strains

Strain[b]	Minimal inhibitory concn (μg/ml) of polymyxin	Rabbit intradermal test	% Survival[a] after 2-h incubation in:	
			NHS	PMNL + 2% NHS
409*	>128	+	86.7 ± 2.8	90.8 ± 2.3
409[s]	<8	+	82.4 ± 3.2	88.7 ± 2.9
C148*	>128	+	57.1 ± 4.1	84.6 ± 4.9
C148[s]	<16	+	47.5 ± 2.8	76.9 ± 3.5
C148[r]	1	+	40.2 ± 4.6	84.8 ± 4.1
A77*	1	−	0	0
A77[p]	250	−	0	0
A75*	0.5	−	0	0
A75[p]	250	−	0	0
O78*	8	−	3.2 ± 1.5	7.2 ± 1.6
O78[p]	250	−	2.8 ± 1.2	6.8 ± 2.9

[a] Average of three separate experiments ± standard deviation.

[b] Strains with ≥50% survival in 50% NHS or in PMNL + 2% NHS after 2 h of incubation were considered resistant. Intermediate resistance was 10 to 49% survival. Strains with <10% survival were considered susceptible. *, Parent strains; p, polymyxin-resistant strains; s, polymyxin-sensitive strains; r, rifampin-resistant strain.

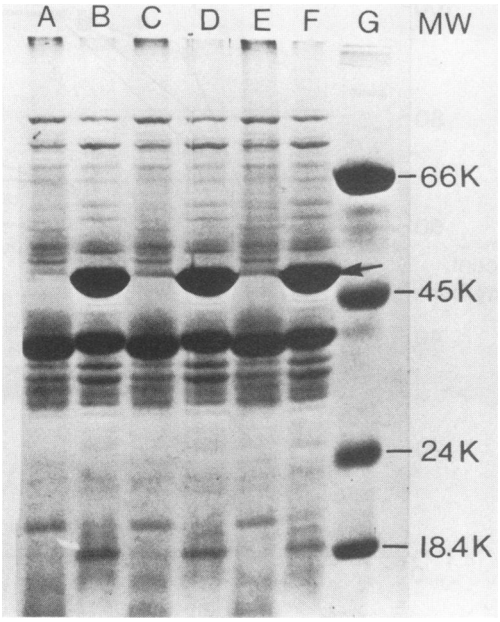

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sarcosinate-insoluble outer membrane proteins of polymyxin-sensitive strains and polymyxin-resistant isogenic strains. The arrow indicates the major protein lost, after acquisition of polymyxin resistance. Lane A, Strain A75^{p}; lane B, strain A75; lane C, strain A77^{p}; lane D, strain A77; lane E, strain O78^{p}; lane F, strain O78. MW, Molecular-weight standards. In lanes A, C, and E are polymyxin-resistant isogenic strains. Acrylamide concentration was 12.5%.

virulent strains. We found that ingestion of these organisms by PMNL was accompanied by degranulation.

Polymyxin resistance and virulence of *H. ducreyi*. Polymyxin-sensitive strains 409^{s} and C148^{s} were obtained from polymyxin-resistant parent strains 409 and C148, respectively. Strain C148^{r} lost its resistance to polymyxin when the strain was made resistant to rifampin. These strains remained virulent for rabbits and resistant to bactericidal activities of NHS and PMNL (Table 1). However, isogenic strains C148^{s} and C148^{r} were less resistant to the bactericidal action of NHS than was the parent strain C148.

In other experiments, polymyxin-resistant strains obtained from polymyxin-sensitive strains by adaptive resistance to polymyxin remained avirulent for rabbits and susceptible to the bactericidal action of NHS and PMNL, as did the parent strains (Table 1).

Analysis of the outer membrane proteins of polymyxin-sensitive strains and the polymyxin-resistant isogenic strains revealed that when resistance to polymyxin was acquired a 47,000-molecular-weight protein was lost (Fig. 4).

DISCUSSION

The pathogenic mechanisms of *H. ducreyi* infections in humans are poorly understood, and relatively few studies on the virulence of this organism have been reported (4, 5, 7, 22). Strains could be discriminated by their ability to produce cutaneous ulcers after intradermal injection in rabbits or humans. Strains which produced skin lesions at the point of inoculation were considered virulent, whereas strains which did not produce lesions were considered avirulent (4, 5, 7). Loss of virulence after repeated laboratory passages has also been reported (4). We have further examined the basis for virulence in these strains and found that virulent strains were resistant to the bactericidal action of normal rabbit serum and NHS and resistant to phagocytosis and killing by human PMNL, in contrast to avirulent strains. The bactericidal activity of normal serum was complement dependent since serum-sensitive strains incubated in heat-inactivated serum survived well within 2 h of incubation at 35°C. Our results also indicate that complement is required for the efficient killing of *H. ducreyi* strains by human PMNL.

Resistance to bactericidal action of serum has been associated with virulence in several gram-negative bacteria (11, 14, 16, 21), most notably as a marker for bacteremic spread. *H. ducreyi* does not produce bacteremia, but resistance to bacteriolysis by normal serum may allow successful extracellular multiplication of the organism within tissues. Similarly, resistance to phagocytosis and killing by human PMNL, an important virulence factor of other gram-negative bacteria (12, 23), was noted with virulent strains of *H. ducreyi*. We propose that resistance to complement-mediated lethal action of serum and resistance to bactericidal activities of human PMNL promote survival and tissue multiplication of *H. ducreyi*, thereby contributing to virulence.

All virulent strains tested were resistant to polymyxin B, whereas avirulent strains were sensitive. This result confirms those reported by Hammond et al. (7). However, we were unable to obtain avirulent strains from virulent strains by one-step mutation that resulted in loss of resistance to polymyxin. Polymyxin-sensitive derivatives from polymyxin-resistant virulent strains remained virulent for rabbits and resistant to the bactericidal activities of NHS and human PMNL.

Acquisition of polymyxin resistance by polymyxin-sensitive avirulent strains by adaptive resistance to polymyxin did not result in acquisition of virulence or resistance to bactericidal activities of serum and PMNL. These results suggest that polymyxin resistance is not directly linked to virulence. Thayer et al. (22) succeeded in making one strain avirulent in vitro after several months of repeated laboratory passages without changing its susceptibility to polymyxin, also an indication that polymyxin resistance may not be associated with virulence. The only phenotypic change which we observed was the loss of a 47,000-molecular-weight protein after acquisition of polymyxin resistance. This protein probably acts as a porin facilitating uptake of polymyxin.

In summary, we have shown that virulent strains of *H. ducreyi* (positive in the rabbit intradermal test) are resistant to bactericidal action of normal serum and to phagocytosis. In addition, we have shown that polymyxin resistance does not constitute a reliable marker for virulence as had been formerly proposed.

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