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Dra/AfaE Adhesin of Uropathogenic Dr/Afa$^+$ *Escherichia coli* Mediates Mortality in Pregnant Rats

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***Escherichia coli* bearing adhesins of the Dr/Afa family frequently causes urogenital infections during pregnancy in humans and has been associated with mortality in pregnant rats. Two components of the adhesin, Dra/AfaE and Dra/AfaD, considered virulence factors, are responsible for bacterial binding and internalization. We hypothesize that gestational mortality caused by Dr/Afa$^+$ *E. coli* is mediated by one of these two proteins, Dra/AfaE or Dra/AfaD. In this study, using *afaE* and/or *afaD* mutants, we investigated the role of the *afaE* and *afaD* genes in the mortality of pregnant rats from intrauterine infection. Sprague-Dawley rats, on the 17th day of pregnancy, were infected with the *E. coli afaE*$^+$ *afaD* and *afaE afaD*$^+$ mutants. The clinical *E. coli* strain (*afaE*$^+$ *afaD*$^+$) and the *afaE afaD* double mutant were used as positive and negative controls, respectively. The mortality rate was evaluated 24 h after infection. The highest maternal mortality was observed in the group infected with the *afaE*$^+$ *afaD*$^+$ strain, followed by the group infected with the *afaE*$^+$ *afaD* strain. The mortality was dose dependent. The *afaE afaD* double mutant did not cause maternal mortality, even with the highest infection dose. The in vivo studies corresponded with the invasion assay, where the *afaE*$^+$ strains were the most invasive (*afaE*$^+$ *afaD* strain > *afaE*$^+$ *afaD*$^+$ strain), while the *afaE* mutant strains (*afaE afaD*$^+$ and *afaE afaD* strains) seemed to be noninvasive. This study shows for the first time that the *afaE* gene coding for the AfaE subunit of Dr/Afa adhesin is involved in the lethal outcome of gestational infection in rats. This lethal effect associated with AfaE correlates with the invasiveness of *afaE*$^+$ *E. coli* strains in vitro.**

Urogenital infections, including asymptomatic bacteriuria, cystitis, and pyelonephritis, are the most common and frequently encountered medical complications of pregnancy and the primary cause of maternal and fetal morbidity and mortality due to infections (23). It has been observed that urogenital infections are associated with low birth weight and preterm labor (20, 23, 29). However, the underlying mechanisms are not yet completely understood.

The most frequent etiologic agent of urogenital infections is uropathogenic *Escherichia coli*, accounting for 65 to 90% of cases (23). Isolates of *E. coli* predominantly associated with gestational urogenital infection include those of the O75 serotype (12). These strains express adhesins of the Dr family that play a critical role in the infectious process and are associated with unique gestational virulence (12). *E. coli* bearing the Dr family of adhesins accounts for 40% of pyelonephritis cases in the third trimester of pregnancy, causes chronic diarrhea in children (50%), and is associated with recurrent urinary tract infection in young adult women (5, 7, 8, 9).

Fimbriae, pili, or adhesins are bacterial structures mediating the adherence to tissues of the human host, which is an important step in the initiation of various infectious diseases (14). Previous studies have shown that uropathogenic *E. coli* expressing Dr/Afa adhesins demonstrated unique renal tissue tropism. Dr/Afa receptors were also found in the colon, lower urogenital tract, ureters, and renal pelvis epithelium. Based on these findings, a receptor adhesin-mediated mechanism of ascending urinary tract infection was postulated (24). The clinical and experimental findings suggest that *E. coli* expressing Dr/Afa adhesins possesses properties that may predispose the establishment of chronic and/or recurrent infections (5, 9).

Dr family or Dr/Afa adhesins consist of the fimbrial adhesins, such as Dr, Dr-II, and F1845, and the afimbrial adhesins AFA-I and AFA-III. These adhesins have similar genetic organizations, an operon consisting of at least five genes. The genes *draA*, *draB*, *draC*, and *draD* (*afaA*, *afaB*, *afaC*, and *afaD*) encoding accessory proteins are highly conservative among all the members of the Dr adhesin family, while the gene *dra*/*afaE*, encoding the adhesin molecule itself, is heterogeneous (14, 24, 25). It has been suggested that the invasive capacity of *E. coli* expressing Dr/Afa adhesins depends on two virulence factors, Dra/AfaE and Dra/AfaD, which are responsible for bacterial binding and internalization (16).

A tissue receptor for uropathogenic Dr/Afa$^+$ *E. coli* is decay-accelerating factor (DAF), which physiologically functions to protect host tissues from cytotoxic effects of complement activation (24, 25). DAF, expressed in human endometrial epithelium, is regulated through the menstrual cycle in a progesterone-dependent manner (17). The DAF expression is also up-regulated during pregnancy and may function as protection

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for the fetus from complement attack (25, 30). Bacterial binding to DAF and internalization into epithelial cells involve Dra/AfaE (9, 16). It has also been suggested that β1 integrin might be a receptor for Dra/AfaD and that additional Dra/AfaD-type-specific receptors exist (28).

Previous in vivo studies of chronic kidney infection in the mouse model and pregnant rat mortality model demonstrated that Dr adhesin is essential for *E. coli* virulence (3, 10, 26). However, the contribution of either the Dra/AfaE or the Dra/AfaD subunit to the infectious process is still poorly understood. In order to develop effective interventions, the mechanisms of virulence of Dr^+ *E. coli* need to be better understood. Therefore, the aim of the study was to analyze Dra/AfaE and/or Dra/AfaD involvement in the pathogenesis of the intrauterine infection using the pregnant rat model.

MATERIALS AND METHODS

E. coli A30 is a clinical isolate bearing AFA-III (19). A30 derivatives containing mutated gene *afaE3*, *afaD3*, or both *afaE3* and *afaD3* were constructed by allelic exchange as previously described (6, 28).

AL858 (*afaE afaD*$^+$). The pILL1131 recombinant plasmid that resulted from the cloning of the *afaE3* gene of A30 interrupted by the *cat* cartridge, into the suicide plasmid pGP704, conferred ampicillin resistance to its host. Filter matings were performed between the kanamycin-resistant strain SM10 (λ *pir*), harboring pILL1131, as a donor and a rifampin mutant of A30 as a recipient. By screening rifampin- and chloramphenicol-resistant transconjugants for ampicillin and kanamycin susceptibility, one clone (AL858) was found to have lost the ability to agglutinate human red blood cells. Hybridization experiments demonstrated that AL858 resulted from a double-crossover event leading to the insertion of a single copy of the *cat*/BamHI cartridge into the *afaE3* gene of A30.

AL861 (*afaE*$^+$ *afaD*). The pILL1238 recombinant plasmid resulted from the cloning of the 2.6-kb PstI fragment of pILL61 (21), containing the *afaD* gene of A30 interrupted by the insertion of the nonpolar kanamycin cassette (22) into the suicide plasmid pGP704 that conferred ampicillin resistance to its host. Conjugation was performed between SM10 (λ *pir*) harboring pILL1238 (donor) and a rifampin mutant of A30 (AL852; recipient). After several subcultures, rifampin- and ampicillin-resistant transconjugants were screened for the expression of kanamycin resistance and loss of ampicillin resistance. One such clone (AL861) was found to carry an interrupted *afaD* gene and an intact *afaE* gene by PCR. AL861 exhibited the same mannose-resistant hemagglutination and adhesin properties as the parental A30 strain.

AL863 (*afaE afaD*). The *afaD3*-mutated derivative of AL858 was obtained in a manner similar to that for AL861. PCR analyses confirmed that AL863 carried mutations in the *afaE3* and *afaD3* genes.

The growth rates were similar for all these strains.

Animal model. Timed-pregnancy Sprague-Dawley rats were obtained from Harlan Sprague Dawley, Houston, TX. Two days before experimental infection, each animal received one dose of streptomycin (7.0 mg/g of body weight) to eliminate possible sites of infection in the urogenital tract that may occur naturally.

Animals were infected with clinical *E. coli* strain A30 (*afaE*$^+$ *afaD*$^+$) or one of its isogenic mutants, AL861 (*afaE*$^+$ *afaD*), AL858 (*afaE afaD*$^+$), or AL863 (*afaE afaD*). An inoculum of *E. coli* in a volume of 200 μl was placed through the cervical os in the cavity of the left uterine horn of each pregnant rat on the 17th day of gestation using a blunted 16-gauge animal feeding stainless-steel needle (Popper & Sons, Inc., New Hyde Park, NY). To determine the relevance of the dose-versus-infection outcome, three different inoculum doses were used at an optical density at 600 nm (OD_{600}) of 2.7 (5×10^{12} CFU/ml), an OD_{600} of 2.0 (5×10^{10} CFU/ml), and an OD_{600} of 0.8 (5×10^{8} CFU/ml). The maternal mortality was evaluated 24 h postinfection. Uteri and kidneys from surviving animals were collected for quantitative bacterial cultures. Tissues were homogenized in phosphate-buffered saline (PBS; pH 7.2), 10-fold dilutions were prepared, and 50-μl aliquots were cultured on L agar and MacConkey plates. The colonization rate was expressed as the number of CFU per gram of tissue.

Invasion assay. Bacterial invasion into HeLa cells was tested by gentamicin protection assay as described earlier (9, 28). Briefly, HeLa cells (~10,000/well) were seeded in 12-well plates and allowed to grow to 75 to 80% subconfluency for 24 h in minimum essential medium (MEM) supplemented with 10% (vol/vol) fetal bovine serum. The monolayer was washed once with PBS, and fresh MEM without fetal bovine serum was added. Fresh 18-h bacterial cultures grown on L agar were used to prepare suspensions in PBS adjusted to an OD_{600} of 2.0 (5×10^{10} CFU/ml), and 1 μl of bacterial suspension was added per 1 ml of MEM. After incubation for 3 h at 37°C with 5% CO_2, the bacterial suspension was removed, and the monolayer was washed twice with medium containing gentamicin (200 μg/ml) and incubated in the same medium for an additional 1 h to kill the extracellular bacteria and select for the internalized bacteria. The monolayers were then washed twice with sterile PBS and incubated for 10 min at room temperature with 1 ml of lysis buffer (0.9% NaCl, 0.1% sodium dodecyl sulfate). About 20 μl of the lysate was plated on L agar plates in duplicate and incubated overnight at 37°C with 5% CO_2. On the following day, the CFU were counted, and the results were expressed as CFU/well. The assay was run in triplicate and repeated three times.

Statistical analysis. Statistical analysis was performed with the GraphPad Prism 4 program using the Mann-Whitney U test and the chi-square test, and *P* values of less than 0.05 were considered significant.

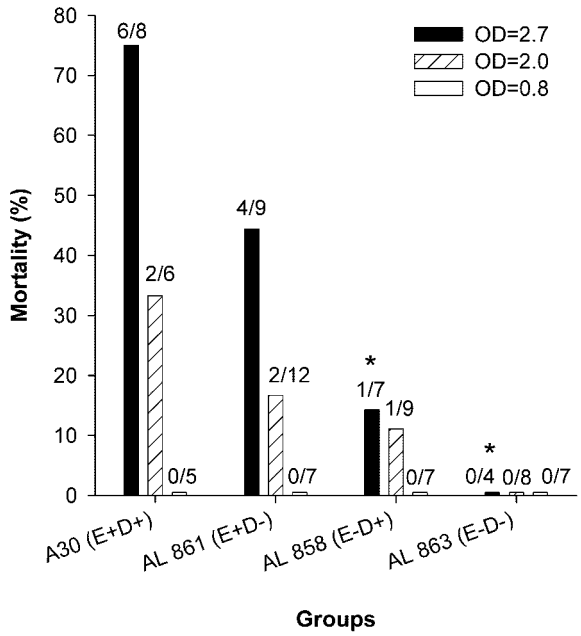

FIG. 1. Maternal mortality in animals infected with clinical strain *E. coli* A30 and its isogenic mutants. Three different concentrations of bacteria, 5×10^{12} CFU/ml, 5×10^{10} CFU/ml, and 5×10^{8} CFU/ml, were used for infection. Bars with asterisks are significantly different from others with the same OD ($P < 0.01$; chi-square test).

RESULTS

Maternal mortality (animal model). In order to investigate the impact of Dr^+ *E. coli* virulence factors AfaE and AfaD on the outcome of intrauterine infection, we assessed maternal morbidity and mortality in pregnant rats infected with the isogenic mutants of *E. coli* expressing a single virulence factor (the *afaE*$^+$ *afaD* and *afaE afaD*$^+$ strains). The clinical strain of *E. coli* expressing both virulence factors (the *afaE*$^+$ *afaD*$^+$ strain) and the double mutant lacking both virulence factors (the *afaE afaD* mutant) were used as positive and negative controls, respectively.

The highest mortality rate was observed in groups infected with *E. coli* expressing the AfaE adhesin in combination with AfaD, strain A30 (*afaE*$^+$ *afaD*$^+$), or expressing the AfaE adhesin alone, strain AL 861 (*afaE*$^+$ *afaD*) (Fig. 1). The differences in mortality between groups infected with the clinical strain and mutants lacking AfaE (the *afaE afaD*$^+$ and *afaE*

afaD mutants) were significant in animals infected with the highest concentration of bacteria at an OD_{600} of 2.7 (A30 versus AL858, $P = 0.0187$; A30 versus AL863, $P = 0.0143$). The difference in mortality between the AL861 (*afaE*$^+$ *afaD*) group and the group infected with the *afaE*$^+$ *afaD*$^+$ clinical strain was not significant, irrespective of the concentration of bacteria used for infection, suggesting that AfaE is a major lethal virulence factor of Afa/Dr$^+$ *E. coli*.

The significance of the dose of infecting agent in the experimental infection outcome was evaluated using three different inocula of bacteria at an OD_{600} of 2.7 (5×10^{12} CFU/ml), an OD_{600} of 2.0 (5×10^{10} CFU/ml), and an OD_{600} of 0.8 (5×10^{8} CFU/ml). Maternal mortality was observed in animals infected with the higher-dose inocula (those with OD_{600}s of 2.7 and 2.0) of bacteria expressing AfaE and/or AfaD and was dose dependent. There was no mortality in the group infected with the *afaE afaD* double mutant, AL863, irrespective of the concentration of bacteria used for infection (Fig. 1).

Quantitative analysis of tissue infection rate. In order to investigate the contribution of *E. coli* virulence factors AfaE and/or AfaD to intrauterine infection during pregnancy, we quantitated the infection in the uteri and kidneys of the infected animals. Due to postmortem effects on bacterial growth, quantitative tissue cultures of dead animals were not performed.

In all tissues, irrespective of the concentration of bacteria used for infection, the clinical strain of *E. coli* (A30) expressing both virulence factors AfaE and AfaD had the highest infection rate. The differences in infection rate between the groups were most pronounced in animals infected with the dose corresponding to an OD_{600} of 2.0 (Fig. 2). In rats infected with single mutants AL861 (*afaE*$^+$ *afaD*) and AL858 (*afaE afaD*$^+$) and the double mutant AL863 (*afaE afaD*), the infection rate was much lower, and the difference reached statistical significance in the uteri but not in the kidneys. However, in the kidneys of animals infected with the double mutant, the infection rate was lower than in those infected with single mutant infections and reached statistical significance compared to animals infected with the *afaE*$^+$ *afaD* strain but not the *afaE afaD*$^+$ strain.

Invasion assay. To better understand the role of AfaE and AfaD virulence factors in the infectious process in vivo, we attempted to evaluate the potential invasive capacity of the clinical strain of *E. coli* and its isogenic mutants. We used a gentamicin protection assay, which allows the assessment of *E. coli* invasiveness since gentamicin is unable to kill intracellular bacteria as opposed to extracellular bacteria, which are killed by bactericidal concentrations of this antibiotic (2, 15). All strains used in the experiment were unable to grow on agar plates after 1 h of incubation in MEM containing gentamicin at a working concentration of 200 μg/ml. There was no bacterial growth, and only a few colonies were found after plating the last PBS washings collected prior to the lysis of the monolayer. The results of bacterial internalization into HeLa cells were expressed as the number of recovered colonies per well.

The internalization rates of both AfaE-positive strains (the *afaE*$^+$ *afaD*$^+$ and *afaE*$^+$ *afaD* strains) were at the level of 10^4 CFU/well and were significantly higher than those in strains which did not express AfaE ($P < 0.01$). This might suggest that AfaE is mostly responsible for the binding and internalization of bacteria and that mutation of the *afaE* gene results in the loss of attachment/invasion properties of this strain. It is interesting to note, however, that the highest internalization rate was observed in the AL861 (*afaE*$^+$ *afaD*) strain and that the difference was significant compared to that in the *afaE*$^+$ *afaD*$^+$ clinical strain ($P < 0.01$) (Fig. 3).

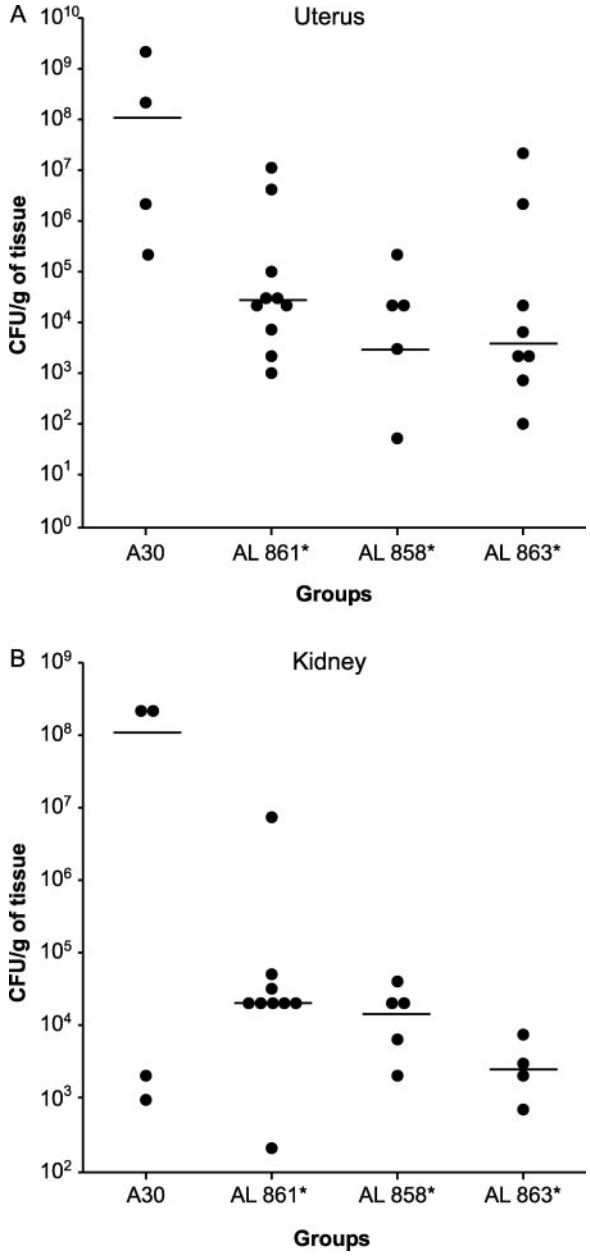

FIG. 2. (A) Infection rate in uteri of animals infected with the clinical strain *E. coli* A30 and its isogenic mutants (at an OD_{600} of 2.0 [5×10^{10} CFU/ml]). (B) Infection rate in kidneys of animals infected with the clinical strain *E. coli* A30 and its isogenic mutants (at an OD_{600} of 2.0 [5×10^{10} CFU/ml]). Results for groups with asterisks are significantly different from those for the A30 group ($P < 0.01$; Mann-Whitney test).

DISCUSSION

Our previous in vivo studies have shown that the *dr*/*afa* cluster is necessary for the virulence of *E. coli*. Using the C3H/HeJ mouse model, we have demonstrated the important role of Dr adhesins in the chronic renal colonization by Dr$^+$ *E.*

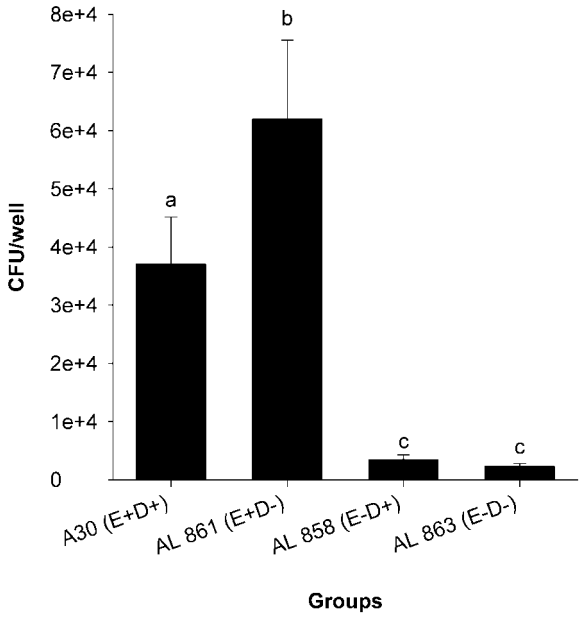

FIG. 3. Invasion of wild-type *E. coli* A30 expressing Dr/Afa adhesins and its isogenic mutants into HeLa epithelial cells in a gentamicin protection assay. Bars marked with different letters are significantly different from one another ($P < 0.01$; Mann-Whitney test).

coli (10), and the rat model was used to demonstrate the importance of Dr adhesins in intrauterine infection during pregnancy (3, 4, 26, 27). Similar sensitivities to Dr^+ *E. coli* infection between pregnant rats and lipopolysaccharide nonresponder C3H/HeJ mice were due to the fact that pregnant animals are to some extent immunocompromised. The change in immune responses may result from the increase in progesterone level during pregnancy, which modifies maternal immune responses in order to protect semiallogenic fetal antigens from rejection (17, 18, 25, 30). In our previous studies, we have also shown that the increased progesterone level might encourage infection with Dr/Afa^+ *E. coli*, up-regulating the expression of DAF, which acts as an epithelial receptor for this bacterium (17, 18).

All our former studies investigated the virulence of *E. coli* with the intact Dr/Afa adhesins. In contrast, the present study illustrates the individual importance of two components of the Dr adhesin, AfaE and AfaD, in the in vivo infection process. Our results suggest that AfaE is a lethal virulence factor leading to increased mortality in pregnant rats. These results are consistent with our prior study on gestational infection, showing that Dr *E. coli* caused death in 30% of pregnant rats (26).

The *afa-3* gene cluster determines the formation of an afimbrial adhesive sheath that is produced by both uropathogenic and diarrhea-associated *E. coli* strains. This sheath is considered to be composed of two proteins, AfaD and AfaE-III. The AfaE subunits assemble end to end to form thin filaments that may be capped with the AfaD protein (1).

Strains producing the AfaE-III protein were initially described as noninvasive because they do not cause keratoconjunctivitis in guinea pigs (Serény test), a property associated with typical invasive enterobacteria, such as *Shigella* spp. or enteroinvasive *E. coli* (21). Later studies, however, demonstrated the invasiveness of these bacteria into epithelial cells (9, 21). In the present study, we have shown, using a gentamicin protection assay, that *E. coli* expressing AfaE-III but lacking AfaD can still invade HeLa cells. This is consistent with our previous experiments showing the invasiveness of Dr/Afa^+ *E. coli* (9, 11) and suggesting that mutation of the hydrophilic domain II of Dra/AfaE eliminates the capacity of *E. coli* to invade HeLa cells although it does not influence its attachment (1a). We speculate that since the *afaE* mutants were unable to invade HeLa cells, the expression of AfaE is an essential requirement for the internalization process.

It was suggested before that AfaD also mediates the invasion of Dr/Afa^+ *E. coli* using β1 integrin as a receptor (28). However, in our study, the internalization rate of the AfaE-negative strain, bearing AfaD, was very low. We speculate that this process might have been less pronounced in our assay since it requires β1 integrin and that there may be different levels of β1-integrin expression between cell lines. The exact role of AfaD in the infectious process requires further investigation.

Apart from the presence of virulence factors, the dose of infecting agent seems to play an important role as well. The mortality of animals infected with *E. coli* strains bearing at least one virulence factor strongly depended on the dose of bacteria used for infection. However, even the highest dose of the *afaE afaD* double mutant did not have any impact on rat mortality. This suggests that the presence of virulence factor(s) AfaE and/or AfaD plays the key role in the gestational pathogenecity of Dr/Afa^+ *E. coli*. To our knowledge, this is the first study that indicates that AfaE and AfaD independently and/or jointly influence maternal mortality in pregnant rats with *E. coli* intrauterine infections.

In all, infected animal tissues were colonized by bacteria, but the highest colonization was in animals infected with $afaE^+$ $afaD^+$ *E. coli*. It is noteworthy that the tissue infection rates in all animals infected with mutants of *E. coli* were similar and significantly lower than those in the group infected with the $afaE^+$ $afaD^+$ clinical strain. This might suggest that the simultaneous expression of both AfaE and AfaD plays an important role in bacterial spread, colonization, and/or survival in vivo, but further studies are necessary to clarify this phenomenon. The reduced mortality with the $afaE^+$ *afaD* strain compared to that with the $afaE^+$ $afaD^+$ clinical strain may also suggest that AfaD cooperates with AfaE in the infectious process in vivo.

The fact that the infection rates in the uteri of animals infected with the *afaE afaD* double mutant were similar to those of animals infected with the $afaE^+$ *afaD* and *afaE* $afaD^+$ single mutants suggests that pregnant rats were not able to eliminate *E. coli* even if it lacked both virulence factors. An alternative explanation is that this clinical strain has additional virulence mechanisms that are not yet recognized.

Significant differences in rat mortality despite similar numbers of bacterial cells isolated from tissues appear to contradict an old dogma that quantitative analysis of infection rate should be preferential diagnostic information for making clinical decisions. These results suggest that the identification of *E. coli* virulence factors, such as Dr/Afa adhesins, could significantly improve diagnosis and influence infection management. It is especially relevant that according to our previous findings, a vast majority of Dr^+ *E. coli* isolates (90%) were multiantibiotic resistant (13).

Our study provides evidence that AfaE is essential for bacterial internalization in HeLa cells and plays a role of the lethal factor in the infectious process in vivo, causing increased maternal mortality. The AfaE-mediated mortality is a very surprising phenomenon, unknown for other adherence factors, and raises the questions of why and how Dra/AfaE$^+$ *E.coli* may confer a lethal outcome in pregnant rats.

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