

## Differences between *Taylorella equigenitalis* Strains in Their Invasion of and Replication in Cultured Cells

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**The ability of *Taylorella equigenitalis*, the causative agent of contagious equine metritis, to invade and replicate in equine derm cells was studied. The kinetics of invasion and replication were determined for four *T. equigenitalis* strains. On the basis of these experiments, a simpler assay in which the invasive as well as the replicative properties of a particular strain could be determined was developed. This assay was used to characterize 32 strains, which had previously been typed by field inversion gel electrophoresis of genomic restriction fragments. The invasiveness of *T. equigenitalis* strains ranged from 3 to 0.015 bacteria per cell and seemed to be associated with the contagiousness of the infection. The replication index (number of intracellular bacteria per cell at 24 h after inoculation divided by the number of intracellular bacteria per cell at 4 h after inoculation) varied from 1 to 857 and seemed to be associated with the severity of the symptoms of contagious equine metritis. There was no association between the invasiveness and the replication index of the strains, nor was there an association of invasion and replication with field inversion gel electrophoresis grouping.**

Contagious equine metritis (CEM) is a venereal disease of horses caused by the bacterium *Taylorella equigenitalis*. Infected stallions are without symptoms, but in mares clinical signs of infection are vaginal discharge, infertility, or early abortion. The principal lesions of CEM are formed in the uterus (12, 14). They are characterized by destruction of endometrial epithelial cells and infiltration of neutrophils into the epithelium and lamina propria of the uterus. Histopathological studies have demonstrated that *T. equigenitalis* adheres to the cilia of the epithelial cells and proliferates on the endometrium (11, 20); internalized bacteria were not observed.

Many pathogenic bacteria adhere to the surface of cells of the host and subsequently become internalized (5). This process, called microbial invasion, can be studied in vitro by measuring bacterial entry into cultured mammalian cells (5, 7). A preliminary microscopic study indicated that *T. equigenitalis* was able to adhere to equine derm (E. derm) cells.

In this study, we describe the kinetics of adherence and invasion and the intracellular replication of *T. equigenitalis*. Four strains were extensively tested in an E. derm cell model. On the basis of these experiments, a simpler assay was developed.

In an earlier study (1), we demonstrated that *T. equigenitalis* strains can be divided into groups by genomic DNA digestion with the restriction enzyme *ApaI* in conjunction with field inversion gel electrophoresis (FIGE). A total of 32 *T. equigenitalis* strains were characterized, and some strains showed similar patterns, which correlated with epidemiological data.

Here, we characterize these strains in a combined invasion and replication assay and associate the results with the clinical properties of the strains.

### MATERIALS AND METHODS

**Bacterial strains.** The origins of the strains studied are given in Table 1. The bacteria were grown on Colombia chocolate agar (1) from frozen stocks and were passaged twice before use in the standard invasion assay.

**Cell culture.** The E. derm cell line, derived from the dermis of a 4-year-old quarter horse mare, was obtained from the American Type Culture Collection (ATCC CCL 57). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (10,000 U/liter), and streptomycin (10 mg/liter). The 24th to 34th serial passages were used.

**Invasion assay.** The invasion assay was performed in 35-mm tissue culture dishes. The cells were seeded at a density of  $2 \times 10^5$  cells per well. Confluent monolayers, containing approximately  $8 \times 10^5$  E. derm cells, were obtained after 48 h. They were washed with DMEM without supplements 30 min prior to inoculation. A bacterial suspension in DMEM was added to each dish. The ratio of added bacteria to tissue culture cells was about 1,000:1. To determine the kinetics of adherence and invasion, the inoculated monolayers (two per time point) were incubated at 37°C with 5% CO<sub>2</sub>-95% air for 0.5, 1, 2, or 4 h. (In final experiments, only a 1-h incubation period was used to allow bacteria to adhere to and invade the cells.) Thereafter, the inoculated E. derm cells were washed five times with DMEM to remove most of the extracellular bacteria. DMEM containing gentamicin (100 mg/liter) was added, and then the cells were incubated for 3 h to kill extracellular bacteria. All *T. equigenitalis* strains isolated so far are gentamicin susceptible (our data) (4, 16). The cells were washed three times with phosphate-buffered saline (PBS) and lysed in a 1% saponin solution in PBS. The number of viable intracellular *T. equigenitalis* bacteria was determined by plating dilutions of this lysate in duplicate on Columbia chocolate agar plates and counting colonies. The average number of bacteria per cell was calculated as the total number of intracellular bacteria divided by the total number of cells. *Escherichia coli* JM 109 served as a noninvasive control in all invasion experiments. The type strain of *T. equigenitalis* (NCTC 11184) was included in every assay to check the reproducibility of the test results.

**Intracellular replication assay.** The intracellular replication assay was performed in parallel with the invasion assay with the same E. derm cells and bacterial suspensions. Bacteria were allowed to invade E. derm cells for 1 h; then gentamicin was added and incubation was continued for 3 h. After the incubation with gentamicin, however, the cells were washed three times with DMEM, fresh medium was added, and incubation at 37°C was continued for 20 h. The intracellular replication assay is essentially the same as the invasion assay with the exception of an extra 20-h incubation period at 37°C after the gentamicin treatment. The supernatant and the cell lysate were subsequently plated onto Columbia chocolate plates and counted as in the invasion assay. Although *T. equigenitalis* is not able to replicate in DMEM culture medium, about 10% of the total bacteria could be detected in the supernatant (8% of the type strain and 20% of strain L68722-2). This is probably due to lysis of E. derm cells. The number of bacteria in the supernatant plus the number in the cell lysate was considered the total number of intracellular bacteria. If bacteria replicated intracellularly, the total number should be higher than the number at the 4-h time

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TABLE 1. History and properties of *T. equigenitalis* strains

Strain <sup>a</sup>	Country of origin	Yr isolated	Breed	FIGE group	No. of bacteria per cell after:		Replication index <sup>b</sup>
					1 h of invasion, 3 h of gentamicin	1 h of invasion, 3 h of gentamicin, 20 h of incubation	
NCTC 11184 <sup>c</sup>	England	1977	Thoroughbred	A	2.10	415	198
L81178 <sup>c</sup>	Ireland	1977	Thoroughbred	A	0.46	45	98
N217/79	England	1979	Thoroughbred	A	0.19	2.5	13
N203/79	United States	1979	Thoroughbred	A	3.00	123	41
N206/79	Australia	1979	Thoroughbred	A	0.35	300	857
N480/82	England	1982	Thoroughbred	A	0.42	0.47	1
N202/79	Belgium	1978	Non-Thoroughbred	B	0.60	3.8	6
N210/79	United States	1979	Thoroughbred?	B	0.67	9.5	14
L10783	The Netherlands	1985	Dutch Warmblood	B	0.18	9.4	52
N415/82	Austria	1982	Non-Thoroughbred	C	0.08	3.4	43
N412/82	Austria	1982	Non-Thoroughbred	C	0.12	27.5	229
N610/88	Switzerland	1988	Non-Thoroughbred	C	0.50	77.5	155
N211/79	Germany	1979	Non-Thoroughbred	D	0.32	20	63
L24902	The Netherlands	1986	Dutch Warmblood	D	0.45	8	18
L46960 <sup>d</sup>	The Netherlands	1987	Dutch Warmblood	D	0.34	32.5	96
L50354 <sup>d</sup>	The Netherlands	1987	Dutch Warmblood	D	0.37	18.8	51
L48987 <sup>e</sup>	The Netherlands	1987	Dutch Warmblood	D	0.036	3.4	94
L50353 <sup>e</sup>	The Netherlands	1987	Dutch Warmblood	D	0.06	1.8	30
L60219 <sup>e</sup>	The Netherlands	1988	Dutch Warmblood	D	0.015	0.25	17
L68722-1	The Netherlands	1988	Hafflinger	D	0.75	2.9	4
L68138	The Netherlands	1988	Hafflinger	D	0.58	5.6	10
L52721 <sup>c,f</sup>	The Netherlands	1987	Dutch Warmblood	E	0.045	0.7	16
L67215	The Netherlands	1988	Trotter	E	0.04	3.4	85
L67865-2	The Netherlands	1988	Trotter	E	0.18	1.4	8
L67865-3	The Netherlands	1988	Trotter	E	0.50	12.3	25
L68338-1	The Netherlands	1988	Trotter	E	0.45	6.3	14
L68338-2	The Netherlands	1988	Trotter	E	0.23	90	391
L68682-1	The Netherlands	1988	Trotter	E	0.087	2.9	33
L68722-2 <sup>c</sup>	The Netherlands	1988	Trotter	E	0.043	5	116
L68722-3	The Netherlands	1988	Trotter	E	0.47	43.5	93
L68722-4	The Netherlands	1988	Trotter	E	0.37	42.5	115
L71205	The Netherlands	1988	Trotter	E	0.22	42.5	193

<sup>a</sup> Strain numbers refer to the strain collection of the Equine Research Station, Newmarket, United Kingdom (N), or the Institute for Animal Science and Health, Lelystad, The Netherlands (L).

<sup>b</sup> Number of intracellular bacteria per cell 24 h after inoculation divided by number of intracellular bacteria per cell 4 h after inoculation.

<sup>c</sup> Strains used to determine the kinetics of invasion.

<sup>d</sup> CEM outbreak 1 among Dutch Warmblood horses in The Netherlands.

<sup>e</sup> CEM outbreak 2 among Dutch Warmblood horses in The Netherlands.

<sup>f</sup> CEM outbreak 3 among Dutch Warmblood horses in The Netherlands.

point. We calculated a so-called replication index as follows: total number of intracellular bacteria per cell at 24 h after inoculation divided by number of intracellular bacteria per cell at 4 h after inoculation (derived from the parallel invasion assay).

**Microscopic examination.** The invasion and replication assays were performed on monolayers grown on a Thermanox coverslip (Nunc, Inc., Roskilde, Denmark). Inoculated monolayers were washed three times with PBS, fixed with ice-cold methanol, stained with Giemsa stain, and examined by light microscopy.

## RESULTS

**Invasion of *E. derm* cells.** The kinetics of invasion were determined for four different *T. equigenitalis* strains: type strain NCTC 11184, strain L81178 from Ireland, and strains L68722-2 and L52721 from The Netherlands (Fig. 1). The type strain and the Irish strain, which belong to FIGE group A, invade the cells more efficiently than the Dutch strains, which belong to FIGE group E. After 1 h, an average of 2 bacteria of the type strain have invaded a cell versus 0.043 and 0.045

bacteria of the two Dutch strains tested. Three repetitions of these experiments confirmed these differences.

**Replication in *E. derm* cells.** Results with the four *T. equigenitalis* strains are shown in Table 1. These strains differed markedly in ability to replicate in the cell. The number of bacteria of the type strain had increased by a factor of 198; that of the Irish strain, by a factor of 98; that of the Dutch strain L68722-2, by a factor of 116; and that of the Dutch strain L52721, by a factor of 16. The difference in numbers of intracellular bacteria of the type strain and Dutch strain L52721 is also demonstrated in the Giemsa-stained infected cells (Fig. 2).

**Combined assay for invasion and replication.** The kinetics of invasion (Fig. 1) show that differences in invasiveness between strains are already obvious after a 1-h invasion period. In the replication assay, the number of intracellular bacteria per cell is determined after a 1-h invasion period and after an incubation of 24 h (1 h of invasion followed by 3 h of gentamicin treatment and 20 h of incubation). Thus, in one assay

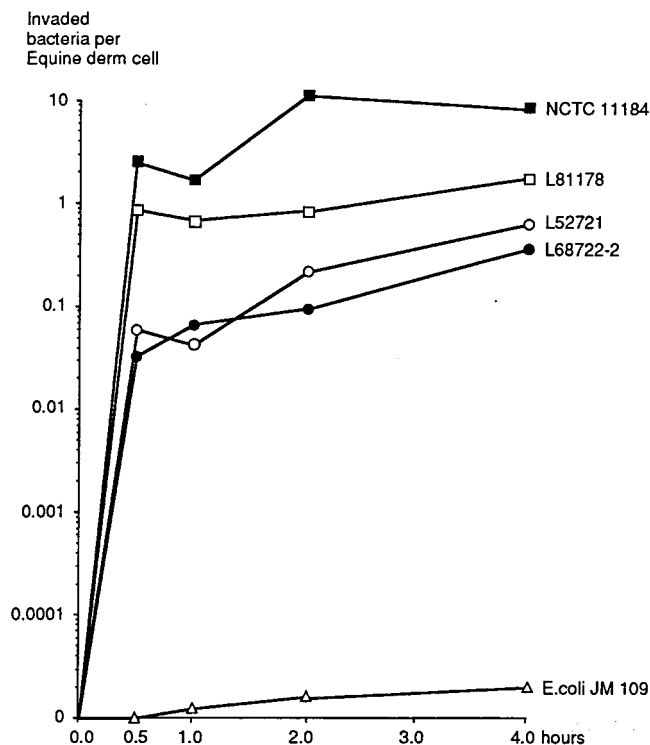


FIG. 1. Kinetics of invasion of E. derm cells by four *T. equigenitalis* strains. Bacteria were added to the cells at 0 h. At the times indicated on the x axis, gentamicin was added. The graph shows the number of viable intracellular bacteria recovered after 3 h of exposure to this antibiotic.

with duplicate plates, the ability of a *T. equigenitalis* strain to invade and replicate can be determined.

**Screening of 32 *T. equigenitalis* strains.** The type strain and strain N203/79 from the United States, both belonging to FIGE group A, were the most invasive strains (3 bacteria per cell). The least invasive strain was L60219 from The Netherlands, FIGE group D (0.015 bacterium per cell [Table 1]).

The Australian strain N206/79, FIGE group A, had the highest replication index; the British strain N480/82, also belonging to FIGE group A, had the lowest replication index.

There was no association between invasiveness and the replication index of the strains. Furthermore, there was no association between FIGE grouping and the invasive and replicative abilities of the strains.

**Reproducibility of results after serial subculturing of strains.** The kinetics of invasion and the intracellular replication index were determined for the type strain and the Dutch strain L68722-2 after two and ten serial passages on Columbia blood agar plates. In this experiment, the numbers of intracellular bacteria after 1 h of invasion were 2.13 and 2.6, respectively, for both passages of the type strain and 0.028 and 0.032, respectively, for both passages of strain L68722-2. Replication indices were 187 and 161, respectively, for both passages of the type strain and 62 and 82, respectively, for both passages of strain L68722-2.

## DISCUSSION

This is the first study that demonstrates that *T. equigenitalis* is able to invade and replicate in cultured cells. *T. equigenitalis* quickly adheres to and invades E. derm cells. In vivo, the bacteria probably require rapid adherence to the genital mu-

cosa to avoid being flushed away. Kanemaru et al. demonstrated in vivo that adherence to cilia of epithelial cells occurs by means of fimbriae (11), although fimbriae have not yet been observed in vitro. If present, fimbriae may well be involved in the adherence of *T. equigenitalis* to E. derm cells.

Invasion levels of *T. equigenitalis* strains differ considerably. Strains from the same outbreak, however, invade E. derm cells at similar levels. The strains isolated from two separate outbreaks in Dutch Warmblood horses in 1987 (L46960 and L50354 versus L48987, L50353, and L60219) (18) were clearly different in these assays.

CEM was initially described in the United Kingdom in 1977 (3). In this first recorded outbreak at a large Thoroughbred stud farm, 6 stallions and 209 visiting mares were involved. After being serviced, 40% of the mares showed clinical signs of infection. The strain of *T. equigenitalis* involved (type strain) and another early isolate (L81178) were considered highly contagious (13, 17) and, interestingly, showed high invasion levels in our assay.

In the first three separate outbreaks in The Netherlands among Dutch Warmblood horses, 3 stallions and 257 visiting mares were involved and only 5 mares were found to be infected (18). The *T. equigenitalis* strains from outbreak 2 (L24902, L46960, and L50354) and outbreak 3 (L52721) had invasion levels that were about 50 times lower than that of the type strain. These observations suggest an association between the ability of *T. equigenitalis* to invade E. derm cells (in vitro) and the contagiousness of the infection (in vivo).

The intracellular replication indices of *T. equigenitalis* strains also differed considerably (1 to 857). Notably, the level of invasion is not associated with the ability to replicate in E. derm cells; e.g., the Australian strain N206/79 has the highest replication index, 857, but a moderate invasion level (0.35 bacterium per cell).

Clinical signs of CEM in Thoroughbred horses have become milder since the disease was discovered (18). Among the strains we tested were three strains isolated from Thoroughbred horses in the United Kingdom in 1977, 1979, and 1982. They all belong to FIGE group A and had invasion levels of 2.1, 0.19, and 0.42, respectively. The replication indices of these strains were 198, 13, and 1. This means that the strain from 1977 (type strain) replicated readily in E. derm cells, the 1979 strain (N217/79) barely replicated, and the 1982 strain (N480/82) did not replicate at all. It may well be that the milder clinical signs over the years are associated with a loss of intracellular reproducibility. This is in agreement with observed clinical signs, varying from mild to severe, among trotters in the 1988 outbreak in The Netherlands. All *T. equigenitalis* strains isolated from this outbreak belonged to FIGE group E, and their invasion levels and replication indices also varied considerably.

Subculturing of *T. equigenitalis* does not affect its ability to invade and replicate in E. derm cells, which indicates that these traits are rather stable in vitro. This is in contrast to the observation of Timoney et al. (19) that in vitro passage caused loss of pathogenicity in vivo.

Genetic and molecular aspects of the bacterial entry process have been studied for several groups of bacteria such as *Shigella* (9, 15), *Salmonella* (6), *Yersinia* (6, 10), and *Campylobacter* (21) species and *Listeria monocytogenes* (8). These invasive bacteria use several separate pathways for entry into cultured cells. The mechanism by which *T. equigenitalis* enters an E. derm cell requires further investigation.

The invasive and replicative abilities of the *T. equigenitalis* strains are not associated with each other, which indicates that invasion and intracellular replication are separate biological

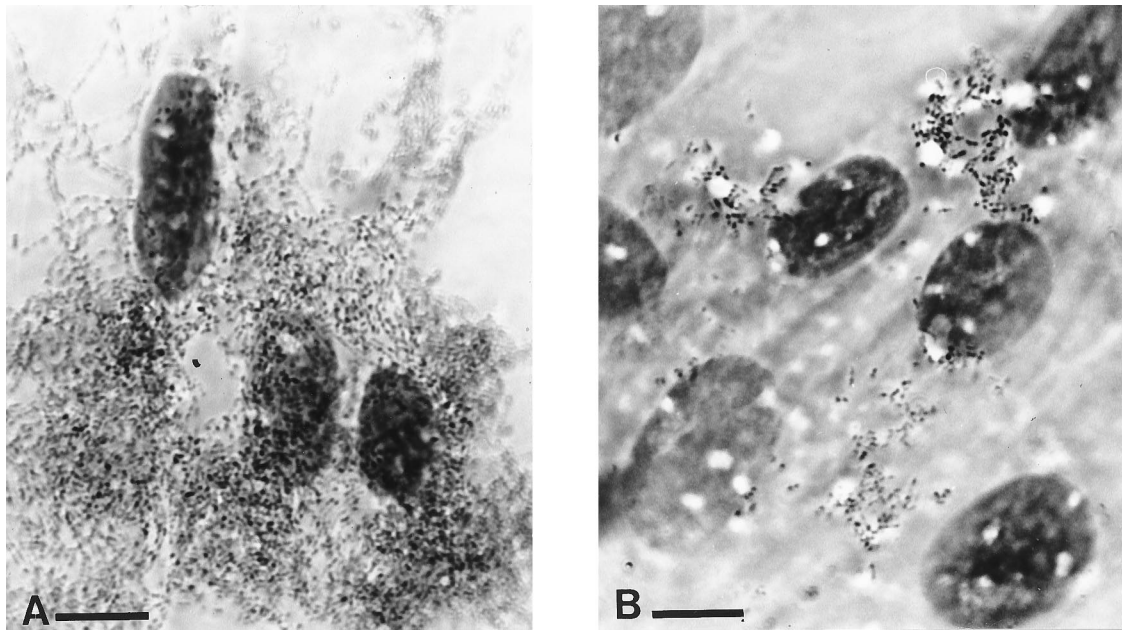


FIG. 2. Intracellular replication assay. E. derm cells were infected by the *T. equigenitalis* type strain (A) or Dutch strain L52721 (B). Bacteria were allowed to invade the cells for 1 h; this was followed by 3 h of gentamicin exposure and 20 h of incubation. The cells were fixed 24 h after inoculation and stained with Giemsa stain. Bar, 10  $\mu$ m.

traits, probably encoded by separate genes. A clinical evaluation of a PCR demonstrates that *T. equigenitalis* is present in 35% of tested healthy horses (2). This means that a considerable number of horses carry *Taylorella* spp. in their genital flora without showing clinical symptoms. The biological differences we found between *Taylorella* strains may relate to virulence. The occurrence of commensal *Taylorella* strains which have no or a low expression of virulence genes could explain the high percentage of PCR-positive horses. In addition, host-associated factors probably also play a role in the pathogenesis of CEM.

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