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Short communication

The extra-intestinal avian pathogenic *Escherichia coli* strain BEN2908 invades avian and human epithelial cells and survives intracellularly

Nathalie Katy Chanteloup [a,*], Gaëlle Porcheron [a], Bernadette Delaleu [b], Pierre Germon [a], Catherine Schouler [a], Maryvonne Moulin-Schouleur [a], Philippe Gilot [a]

[a] INRA, UR1282 Infectiologie Animale et Santé Publique, Laboratoire de Pathogénie Bactérienne, F-37380 Nouzilly, France
[b] INRA, UMR85 Physiologie de la Reproduction et des Comportements, CNRS, Université de Tours, Haras Nationaux, F-37380 Nouzilly, France

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ABSTRACT

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) strains are responsible for a wide range of diseases in humans and animals. Using *in vitro* invasion assays and transmission electron microscopy, we showed that BEN2908, an ExPEC strain of avian origin (also termed APEC for Avian Pathogenic *E. coli*), is able to usurp cellular endocytic pathways to invade A549 human type II pneumocytes and LMH avian hepatocytes where it is able to survive over several days. Although type 1 fimbriae are the major adhesin of BEN2908, proportions of adherent fimbriated or afimbriated bacteria that entered cells were comparable. Internalization of BEN2908 into human pneumocytes reinforces previous studies indicating that APEC strains could represent a zoonotic risk.

1. Introduction

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) strains are responsible for a wide range of diseases in humans and animals, including urinary tract infections, neonatal meningitis, septicaemia and pneumonia (Russo and Johnson, 2003). In avian species, ExPEC strains, also termed APEC for avian pathogenic *E. coli*, are responsible for a systemic disease that starts with a respiratory tract infection and evolves into septicaemia and colonization of internal organs (heart, liver, spleen, etc.). Phylogenetic studies and virulence-factor pattern analysis have shown close relationships between ExPEC strains of human and avian origin, suggesting that APEC strains could represent a zoonotic risk (Rodriguez-Siek et al., 2005; Moulin-Schouleur et al., 2007).

In the present study, to gather new knowledge on the possible zoonotic aspect of APEC, we examined the ability of the APEC strain BEN2908 to enter and survive intracellularly in human type II pneumocytes, an epithelial cell line originating from a target (lung) of early phase of ExPEC infection. As no avian pneumocyte cell lines are available, we then evaluated the ability of BEN2908 to interact with avian hepatocytes, a cell line originating from a target (liver) of late phase of APEC infection.

2. Materials and methods

2.1. Cell lines, bacterial strains and growth conditions

A549 human type II pneumocytes (Giard et al., 1973) and LMH avian hepatocytes (Kawaguchi et al., 1987) were grown with 5% CO_2 at 37 °C in DMEM and at 40 °C in DMEM/Ham's F12 (1:1), respectively. Media were supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum. The APEC strain BEN2908, O2:K1:H5 (*fim*$^+$, *pap*$^-$) is a nalidixic acid-resistant derivative of strain MT78 which was isolated from the trachea of a chicken with respiratory infection (Dho and Lafont, 1982). Strain DM34 is an isogenic mutant of BEN2908 lacking the *fim* operon encoding type 1 fimbriae. Electron microscopic

* Corresponding author. Tel.: +33 2 47 42 76 79; fax: +33 2 47 42 77 74.
E-mail address: Nathalie.Chanteloup@tours.inra.fr (N.K. Chanteloup).

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examination of DM34 revealed only flagella as cell surface appendages (Marc et al., 1998). Bacteria were routinely grown in Luria–Bertani (LB) broth at 37 °C with shaking. Growth of strains BEN2908 and DM34 in LB and cell culture media was found to be similar, under the conditions as described above.

2.2. Invasion, association and survival assays

For invasion assays, A549 and LMH cells were seeded in 24-well plates to reach approximately 2.5×10^5 and 5×10^5 cells per well after 24 h of culture, respectively. Cell monolayers were infected with mid-log-phase bacteria ($OD_{600} \approx 0.350$) at a multiplicity of infection (MOI) of 10 and incubated for 2 h. Cells were washed and remaining extracellular bacteria were killed by incubating for an additional 1 h 30 min in medium containing gentamicin (100 μg/ml). Cells were then washed and lysed with sterile water for 30 min at 4 °C. For association assays, the additional incubation with gentamicin was omitted. Inocula, cell-associated (adherent and intracellular) and intracellular bacteria were enumerated by viable counts on LB agar. To provide a true adhesion value, the number of intracellular bacteria was subtracted from the number of cell-associated bacteria. Adhesion and invasion were respectively calculated as the number of adherent or intracellular bacteria, divided by the number of bacteria in the inoculum, and expressed as a percentage. Invasion efficiency was calculated as the number of intracellular bacteria divided by the number of adherent bacteria, and expressed as a percentage.

For invasion-blocking experiments, inhibitors were added to cell monolayers 30 min prior to inoculation with bacteria and maintained throughout the invasion assay. Chlorpromazine, cytochalasin D, filipin III and nystatine were all purchased from Sigma–Aldrich and stock solutions were prepared in DMSO with the exception of chlorpromazine, which was solubilized in water. The chosen concentration of each inhibitor (0.8 μM cytochalasin D, 14 μM chlorpromazine, 25 μM nystatin and 6 μM filipin III) was the lowest concentration necessary to obtain the maximal inhibitory effect on bacterial uptake. Invasion efficiency was calculated and expressed in relation to that of the untreated controls. Inhibitors and DMSO, at the final concentrations used, were controlled to have no notable effects on the viability and growth of bacteria (as checked by viable counts) or eukaryotic cells [as checked by trypan blue exclusion assay (Longo-Sorbello et al., 2006)]. DMSO at the used concentrations (0.05% for cytochalasin D and nystatin, 0.1% for filipin III) was proven not to interfere with the adhesion of bacteria with eukaryotic cells. Adhesion, invasion and invasion-blocking experiments were conducted in triplicate and were independently repeated at least three times.

For intracellular survival assays, A549 and LMH cells were seeded in 6-well plates to reach 1×10^6 and 2.5×10^5 cells per well after 24 h of culture, respectively. The invasion assay was performed as described above. To delay cell overgrowth after the initial killing of extracellular bacteria with 100 μg/ml of gentamicin, the infected cells were cultured in medium containing 2.5% of serum only. At that time, the gentamicin concentration was reduced to 10 μg/ml (a concentration shown to kill BEN2908) to avoid putative accumulation within cells during prolonged incubation. The medium was changed daily. Intracellular and extracellular bacteria were enumerated by viable counts on LB agar every 24 h until 192 h post-inoculation. Intracellular survival assay was conducted in duplicate and was independently repeated at least two times.

2.3. Transmission electron microscopy (TEM)

At 2 h post-infection, infected A549 and LMH monolayers were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 h at 4 °C. Infected LMH monolayers were also fixed similarly at 72 h post-infection. They were then post-fixed in 2% OsO4 – 1.5% potassium ferrocyanide – 0.1 M cacodylate buffer for 1 h 30 min at room temperature. After dehydration using increasing ethanol concentrations, the monolayers were embedded in Epoxy Resin. Ultrathin sections (70 nm) were collected on copper grids, contrasted with uranyl acetate and lead citrate, and observed with a CM10 electron microscope (Philips, Eindhoven, Netherlands). Analysis software (Soft Imaging System, Germany) was used for image acquisition.

2.4. Statistical analysis

Data were analysed using Student's *t*-test with $P < 0.05$ considered as significant.

3. Results

3.1. Interaction of strain BEN2908 with epithelial cells

The adhesive and invasive abilities of strain BEN2908 were tested on A549 human type II pneumocytes and LMH avian hepatocytes. Fig. 1A (black bars) shows that BEN2908 adhered to LMH and A549 cells to the same extent (366% and 317% of the inoculum, respectively). Bacterial uptake occurred in both cell lines although the internalization of BEN2908 was significantly higher ($P = 0.004$) in LMH cells (23.10% of the inoculum) than in A549 cells (0.756% of the inoculum) (Fig. 1B, black bars). The low internalization level of BEN2908 in A549 cells could not be increased by increasing the MOI from 10 to 100 (data not shown).

The ability of BEN2908 to invade LMH and A549 cells was also examined by transmission electron microscopy. Fig. 2 illustrates evidence of invasion of both cell lines by BEN2908. Intracellular bacteria were observed both singly and in groups and were clearly enclosed in membrane-bound vacuoles (Fig. 2A–D). *E. coli* were observed bound to the cell surface of LMH cells and engulfed by lamellipodia suggesting a zipper-like mechanism of internalization (Fig. 2B, arrowhead). Moreover, some images of synchronized dividing intracellular bacteria suggest that intravacuolar multiplication may occur during infection of LMH cells (Fig. 2C, arrowheads). In infected A549 monolayers, *E. coli* were observed bound to the cell surface and surrounded by microvilli (Fig. 2D and F, arrowheads). In