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Characterization of the invasive and inflammatory traits of oral *Campylobacter rectus* in a murine model of fetoplacental growth restriction and in trophoblast cultures

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ABSTRACT

Campylobacter species (*C. jejuni*, *C. fetus*) are enteric abortifacient bacteria in humans and ungulates. *Campylobacter rectus* is a periodontal pathogen associated with human fetal exposure and adverse pregnancy outcomes including preterm delivery. Experiments in pregnant mice have demonstrated that *C. rectus* can translocate from a distant site of infection to the placenta to induce fetal growth restriction and impair placental development. However, placental tissues from human, small-for-gestational age deliveries have not been reported to harbor *C. rectus* despite evidence of maternal infection and fetal exposure by fetal IgM response. This investigation examined the temporal relationship between the placental translocation of *C. rectus* and the effects on fetal growth in mice. BALB/c mice were infected at gestational day E7.5 to examine placental translocation of *C. rectus* by immunohistology. *C. rectus* significantly decreased fetoplacental weight at E14.5 and at E16.5. *C. rectus* was detected in 63% of placentas at E14.5, but not at E16.5. In *in vitro* trophoblast invasion assays, *C. rectus* was able to effectively invade human trophoblasts (BeWo) but not murine trophoblasts (SM9-1), and showed a trend for more invasiveness than *C. jejuni*. *C. rectus* challenge significantly upregulated both mRNA and protein levels of IL-6 and TNFα in a dose-dependent manner in human trophoblasts, but did not increase cytokine expression in murine cells, suggesting a correlation between invasion and cytokine activation. In conclusion, the trophoblast-invasive trait of *C. rectus* that appears limited to human trophoblasts may play a role in facilitating bacterial translocation and placental inflammation during early gestation.

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1. Introduction

Campylobacter rectus is an exclusively oral Gram-negative, anaerobic and motile bacterium with a wide array of virulence factors including flagellum, surface layer proteins (S-layer), RTX-type toxins, GroELlike proteins and lipopolysaccharide (LPS) (LaGier and Threadgill, 2008; Okuda et al., 1997; Wang et al., 2000). Together with other oral anaerobic bacteria, *C. rectus* is associated with the initiation and progression of periodontal disease (Ihara et al., 2003; Socransky et al., 1998; Tanner et al., 1998). *C. rectus* has been implicated in the association between periodontal disease and adverse pregnancy outcomes. For example, fetal exposure to *C. rectus* has been demonstrated to be higher in preterm than in full term neonates (Madianos et al., 2001). Moreover, *C. rectus* count levels are higher in the oral microbiota of pregnant women with increased salivary estradiol concentrations (Yokoyama et al., 2008). Indeed, *C. rectus* seems to thrive under high concentrations of estradiol and progesterone which have been shown to significantly enhance *C. rectus* growth *in vitro* (Yokoyama et al., 2005). Other *Campylobacter* spp. including *C. fetus* and *C. jejuni* have also been reported to be associated with miscarriages, premature labor and severe perinatal infection in both humans as well as in animals (Allos, 2001; O'Sullivan et al., 1988; Simor et al., 1986; Wong et al., 1990). It is then plausible that *C. rectus* may be an important contributor to adverse pregnancy outcomes due to its ability to disseminate systemically during pregnancy.

Our laboratory has studied the effects of *C. rectus* systemic infection on the fetoplacental unit using a murine model of intra-chamber injection with live bacteria (Yeo et al., 2005). This intra-chamber model demonstrated that remote subcutaneous *C. rectus* maternal infection increases fetal resorptions and induces fetal growth restriction (Offenbacher et al., 2005). *C. rectus* infection also results in abnormal placental architecture, as evidenced by the decreased width of the vascular labyrinth and the increased width of decidual tissue in the placentas of infected growth-restricted mice (Bobetsis et al., 2007). If *C. rectus* disseminates systemically to reach the placenta it is then likely to interact with placental cells that express pattern recognition receptors (i.e., Toll-like receptors) (Abrahams et al., 2004), and subsequently induce a proinflammatory response that ultimately may contribute to an adverse pregnancy outcome. Indeed, recent results from our group have suggested that murine placentas from oral *C. rectus*-infected dams show enhanced placental TLR4 expression along with increased vasodilation in the junctional zone surrounded by focal areas of inflammatory infiltrate (Arce et al., 2009).

The *in vitro* interactions of *C. rectus* with placental cells are yet to be studied. Hypothetically, direct *C. rectus* contact with trophoblasts may alter gene expression and induce a proinflammatory response. *C. rectus* may also have the ability to invade placental trophoblasts since other *Campylobacter* species have been shown to readily invade host or immunocompetent cells, a feature that may play a role in their virulence potential. For example, *C. jejuni* invasion of enterocytes has been shown to induce oncotic changes in these cells with extensive cytoplasmic vacuolation and loss of plasma membrane integrity, an important feature in the pathogenesis of bacterial enteritis (Kalischuk et al., 2007). Moreover, bacterial invasion into mammalian cells has also been proposed as an important mechanism to evade phagocytic immune cells and allow systemic dissemination and bacterial translocation to different tissues (Li et al., 2008; Medina et al., 2003).

In this report we evaluated the presence of *C. rectus* in the placenta of pregnant mice that were infected subcutaneously with live bacteria. We also evaluated the *in vitro* ability of *C. rectus* to invade human as well as murine trophoblast cells, and whether *C. rectus* infection induces changes in two important proinflammatory genes at the messenger RNA and protein levels.

2. Methods

2.1. Mouse model of C. rectus infection

All procedures were in accordance with the animal welfare guidelines and approved by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee. The mouse infection model used was similar to that described before (Yeo et al., 2005). BALB/c mice were housed under controlled and standardized conditions with 12 h light–dark cycles. Regular mouse diet and water were provided *ad libitum*. Females were enrolled in the experiments at approximately 6 weeks of age and immediately had a steel chamber implanted subcutaneously. After 1 month of healing, females were mated overnight with males of the same strain. The next morning, females were removed from the male cages and examined for vaginal plugs. If a plug was found, that day was recorded as embryonic day E0.5. At E7.5, pregnant mice received an intra-chamber injection of 100 μL of 10^9 CFU/mL live *C. rectus* or saline. Mice were then sacrificed at E14.5 and fetuses ($n = 15$ from 3 non-infected dams and $n = 25$ from 4 infected dams) and their respective placental tissues were collected. In preliminary experiments to establish the growth restriction model we collected weight data for fetoplacental units obtained from 27 non-infected dams and 32 infected dams sacrificed at E16.5. For histological analysis, placentas were collected and bisected sagitally then fixed in 4% paraformaldehyde and embedded in paraffin. Sections (6 μm) were stained using standard hematoxylin and eosin protocols and imaged using a Nikon Microphot-FXA Microscope equipped with a QImaging Micropublisher CCD camera. Morphometric measurements of the area occupied by each placental layer, namely decidua, spongiotrophoblast layer and labyrinth, were conducted using the "Image J" software (http://rsb.info.nih.gov/ij/). For detection of *C. rectus* in placental tissues, placentas from 2 control mice ($n = 10$) and 2 infected mice ($n = 11$) from gestational day E14.5 were examined by immunostaining. Briefly, sections were de-paraffinized, re-hydrated in ethanol/H_2O washes and permeabilized by incubation in 0.2% Triton X in PBS. Slides were then incubated for 1 h in blocking buffer (5% BSA, 1% goat serum and 0.2% Triton-X in PBS) and then incubated overnight at 4 °C with an FITC-conjugated anti-*Campylobacter* antibody (Kirkegaard & Perry Labs, MD) and Texas Red-conjugated Phalloidin