Quantifying Translocation of *Listeria monocytogenes* in Rats by Using Urinary Nitric Oxide-Derived Metabolites

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The urinary nitric oxide metabolites NO$_2^-$ and NO$_3^-$ (summed as NOx) are a noninvasive, quantitative biomarker of translocation of salmonella from the intestinal lumen to systemic organs. *Listeria monocytogenes* is a food-borne gram-positive pathogen that can also cross the intestinal epithelium. In this study, we tested the efficacy of urinary NOx as a marker of listeria translocation. Rats (eight per group) were orally infected with increasing doses of *L. monocytogenes*; control rats received heat-killed listeria. The kinetics of urinary NOx and population levels of listeria in feces were determined for 7 days. Another group of rats was killed 1 day after infection to verify translocation by culturing viable listeria from systemic organs. Oral administration of increasing doses of *L. monocytogenes* resulted in a time- and dose-dependent increase in urinary NOx excretion. Translocation was a prerequisite for inducing a NOx response, since heat-killed *L. monocytogenes* did not elevate NOx excretion in urine. Fecal counts of listeria also showed dose and time dependency. Moreover, the number of viable *L. monocytogenes* cells in mesenteric lymph nodes also increased in a dose-dependent manner and correlated with urinary NOx. In conclusion, urinary NOx is a quantitative, noninvasive biomarker of listeria translocation.

[Listeria monocytogenes](https://doi.org/10.1128/AEM.66.12.5301-5305.2000) is a food-borne gram-positive pathogen that is able to translocate across the gut epithelium, resulting in listeriosis, i.e., systemic infection. Most healthy adults experience a limited infection, with at most mild influenza-like symptoms or, in some cases, gastroenteritis (2, 13, 30). However, listeriosis is extremely dangerous for pregnant women, causing abortions and stillbirths, and for newborn, elderly, and immunocompromised individuals, causing meningitis, meningoencephalitis, or sepsis (13, 14, 18, 21, 30). In Europe, the incidence of listeriosis is increasing (2, 14). The rate of mortality caused by listerial infection, excluding abortions, is one of the highest among bacterial infections. Overall fatality rates of 24 to 44% have been reported (2, 14, 18, 21). Because of the property of pathogens to acquire resistance to antibiotics, new approaches to prevent listeriosis deserve attention. Luminal factors such as gastric acidity, antimicrobial bile salts and fatty acids, and pancreatic enzymes contribute to the intestinal non-specific defenses by killing pathogens. Therefore, changing the composition of the diet and thus changing the composition of gastrointestinal contents may affect colonization and translocation of *L. monocytogenes*. To study the efficacy of food components, an animal model of food-derived listeriosis with a quantitative, reliable, and accurate biomarker for translocation is required. Translocation of *L. monocytogenes* is observed in rats (24, 28) and mice (26), making them suitable models. Classically, translocation of listeria in animal models is determined by microbiological culturing of lymphoid organs (24, 26, 28). This approach has some disadvantages. First, culturing of organs is invasive, demanding killing of animals. Consequently, many animals are needed when more than one time point is necessary, e.g., when kinetics of both translocation and colonization have to be determined. Second, microbiological culturing only measures viable bacteria and not those already killed by immune cells and is therefore not representative of the total amount of translocated pathogens. Our laboratory has previously shown that urinary nitrate and nitrite (summed as NOx), which reflects the production of nitric oxide (NO) by phagocytic cells (15), is a quantitative biomarker of salmonella translocation and is useful in studying the efficacy of functional food components on salmonella infection (5, 6, 27). Upon translocation, *L. monocytogenes* infects mesenteric lymph nodes (MLNs), spleen, and liver (24, 26, 28), resulting in infiltration of neutrophils and monocytes (7). These leukocytes, together with hepatocytes, Kupffer cells, and splenic macrophages, are able to induce NO synthase (12, 22, 23, 25, 33). It has been shown that *L. monocytogenes* induces NO synthase in murine spleen cells (33) and macrophages (25) and increases the concentration of NOx in serum and urine when systemically administered in mice (4, 16). Thus, urinary NOx or NO may also be a quantitative biomarker of listeria translocation. Therefore, a strictly controlled experiment was performed with rats that were intragastrically infected with increasing doses of *L. monocytogenes*. Urinary NOx and viable listeria in lymphoid organs and feces were measured.

**MATERIALS AND METHODS**

**Bacterial culturing.** *L. monocytogenes* 4B (clinical isolate, B124 from the collection of our institute) was routinely stored at −80°C in brain heart infusion broth (Difco, Detroit, Mich.) containing 20% (vol/vol) glycerol. Stock solutions were quickly thawed, plated on listeria-selective PALCAM plates (Merck, Darmstadt, Germany), and then incubated aerobically at 37°C for 18 h. Subsequently, a few colonies were inoculated in brain heart infusion broth, followed by overnight incubation at 37°C under aerobic conditions. Bacterial cells were collected by centrifugation (15 min at 3,500 × g), washed three times in sterile saline, and resuspended in saline containing 5% (wt/vol) sodium bicarbonate. The virulence of the strain used was sustained by routine oral passage in Wistar rats, followed by isolation from spleen and liver at day 3 after oral administration.

**Animals and infection.** The experimental protocol was approved by the animal welfare committee of Wageningen University, Wageningen, The Netherlands. Male Wistar rats (specific pathogen free, WU; Harlan, Horst, The Netherlands), 9 weeks old with a body weight of approximately 325 g, were individually housed in metabolic cages. The environmental temperature (22 to 24°C), relative humidity (50 to 60%), and dark-light cycle (light, 0600 to 1800 h) were kept constant. The rats were fed purified diets consisting of 20% casein, 63% glucose,
5% cellulose, 4% corn oil, and vitamins and minerals according to the AIN-93 recommendation (25), except for choline, which was added as choline chloride instead of choline tartrate, and calcium, which was added as calcium phosphate (CaHPO₄·2H₂O; 180-mmol/kg diet) instead of calcium carbonate (125-mmol/kg diet). Diets were supplied as a porridge with 68% dry weight (dry diets mixed with double-distilled water) to minimize food spilling and subsequent contamination of urine and feces. Rats were given free access to food and demineralized drinking water. Food intake and body weight were recorded every 2 to 4 days preinfection and daily postinfection.

After 2 weeks of habituation to diets and housing conditions, four groups of 16 rats were orally infected by gastric gavage with 1 ml of saline containing 3% (wt/vol) sodium bicarbonate with either 8 × 10⁷, 8 × 10⁸, or 8 × 10⁹ viable *L. monocytogenes* cells or 8 × 10⁹ heat-killed *L. monocytogenes* cells (90 min at 60°C). This range is comparable to previously reported doses used in animal models of oral listeria infection (17, 24, 26). Viability and the exact number of *L. monocytogenes* cells in the inocula were determined by plating on PALCAM. Rats were killed by inhalation of carbon dioxide at either day 1 or day 7 after inoculation. Those killed at day 1 of the infection (eight per group) were used to measure translocation by enumerating viable *L. monocytogenes* cells in organs. Their MLNs, spleen, and liver were removed aseptically, weighed, and homogenized (Ultraturrax model Pro 200; Pro Scientific, Inc., Monroe, Conn.) in sterile saline. For counting of *L. monocytogenes* cells, 10-fold dilutions were plated on PALCAM and plates were subsequently incubated aerobically at 37°C for 36 h. The detection limit for tissue homogenates was 1.7 log₁₀ CFU/ml of homogenates, which implies detection limits of 1.7 log₁₀ CFU/MLN, 2.1 log₁₀ CFU/spleen, and 2.2 log₁₀ CFU/g of liver (wt, wet), respectively. The other rats were used to determine translocation and colonization parameters in urine and feces, respectively. Complete 24-h urine samples were collected daily, starting 2 days before infection until the end of the experiment. Oxytetracycline (Sigma, St. Louis, Mo.; approximately 100 times the MIC for most aerobes) was added to the drinking water. Food intake and body weight were recorded every 2 to 4 days per group. DL, detection limit. Values on the same day not sharing the same letter are significantly different (P < 0.05), as determined by ANOVA followed by Student’s t-test with Bonferroni correction.

**RESULTS**

**Rat growth and food intake.** Body weight gain was not affected by the dose of *L. monocytogenes* administered. Mean body weight was 324 g at the start of the experiment, whereas the mean final body weight was 375 g. Food intake was not significantly affected by the dose of *L. monocytogenes* given. Before infection, average food intake was 23.1 g/day. After infection, mean food intake was 21.1 g/day.

**Intestinal colonization and translocation of *Listeria*.** Intestinal colonization was determined by enumerating viable *L. monocytogenes* cells in feces. Figure 1 shows the kinetics of fecal excretion of viable *L. monocytogenes*. On day 1 after inoculation, high levels of listeria were excreted. *L. monocytogenes* excretion declined gradually to the detection limit within 1 week after inoculation. Fecal excretion of viable *L. monocytogenes* was dose dependent. As expected, no viable *L. monocytogenes* could be detected in feces of rats that received 8 × 10⁹ heat-killed bacteria. No clinical signs of diarrhea were observed during the course of infection.

Translocation was measured by counting the number of viable listeria cells in lymphoid organs 1 day after inoculation. MLNs of rats receiving 8 × 10⁹ heat-killed *L. monocytogenes* cells were sterile (Fig. 2). The amount of *L. monocytogenes* cells in MLNs of rats infected with viable bacteria depended on the dose administered. MLN weight was not affected by infection (131 ± 51, 188 ± 40, 127 ± 22, and 109 ± 27 mg for heat-killed listeria at 8 × 10⁷, 8 × 10⁸, and 8 × 10⁹ CFU, respectively).
respectively). No viable pathogens could be detected in the spleens and livers of infected rats.

Figure 3A shows the kinetics of urinary NOx excretion upon challenge with different doses of L. monocytogenes. Before infection, no differences in urinary NOx were observed between treatment groups. Heat-killed L. monocytogenes cells did not affect NOx excretion. Administration of viable L. monocytogenes, however, increased urinary NOx output, starting at day 2 of the infection. Peak values of NOx excretion were observed on days 3 to 4 after inoculation. Urinary NOx output returned to baseline levels on day 6 after infection, irrespective of the dose administered. The total infection-induced NOx excretion increased in a dose-dependent manner (Fig. 3B). The correlation coefficient between mean viable listeria counts in MLNs and the mean total infection-induced NOx per listeria dose was 0.9902 (P < 0.01).

**DISCUSSION**

This study was performed to investigate the efficacy of urinary NOx as a quantitative biomarker of translocation of L. monocytogenes. This food-borne pathogen can colonize the intestinal tract, invade the intestinal mucosa, and spread to systemic organs, causing sepsis and meningitis in individuals with hampered immune function. Colonization of listeria depended on the oral dose (Fig. 1). Our study also showed that the number of viable listeria cells in MLNs increased with higher doses of orally administered L. monocytogenes (Fig. 2). We were, however, unable to detect L. monocytogenes in spleen and liver. This may be explained by the time point at which organs were sampled, (i.e., day 1 after infection). Although Hirose et al. (17) described that dissimination of L. monocytogenes to spleen and liver can be detected on day 1 after intragastric inoculation of 3 x 10⁹ CFU in male F344/Slc rats, other oral models using mice showed that this pathogen can be detected in the spleen only from the second day and beyond (24, 26). Species and strain differences (F344/Slc versus Wistar rats in our study) and, probably, the distinct diets (rodent chow versus purified diets in our study) may account for this discrepancy. Literature data on the kinetics of translocation to MLNs are consistent; all studies showed translocation to MLNs on day 1 after inoculation (17, 24, 26). Although the time of spreading to spleen and liver may depend on the animal model used, these studies as well as our experiment clearly show that extraintestinal dissemination of L. monocytogenes occurs in a dose-dependent manner in both rats and mice (26, 30).

NO synthesis is a primary reaction of phagocytic cells to microbes (19, 23) or bacterial components such as lipopolysaccharide (19, 31) and is known to respond in a dose-dependent manner (3, 19). NO is rapidly oxidized to NO₂⁻ and NO₃⁻, which are excreted in urine (15). Our laboratory has previously shown that urinary NOx is a more discriminative biomarker for salmonella translocation than enumeration of systemic bacteria from organs (27). Analysis of urinary NOx is also noninvasive, not requiring killing of the animals. L. monocytogenes is also known to stimulate NO production in murine peritoneal macrophages (25) and spleen cells in vitro (33). In vivo studies with mice have shown that systemically administered L. monocytogenes increases NO in blood and urine in a dose-dependent manner (4, 16). Using the NO synthase inhibitor N⁶-monomethyl-L-arginine, Boockvar et al. (4) proved that listeria-mediated urinary NOx was derived from induced...
NO synthase activity. Our study showed that orally administered *L. monocytogenes* provoked urinary NOx excretion in rats. Values peaked at days 3 and 4 of the infection and gradually declined thereafter, with baseline levels reached at day 6. These kinetics are remarkably consistent with the kinetics reported for bacterial counts in lymphoid organs (24, 26) after oral listeria infection and with the kinetics of inducible NO synthase mRNA in murine peritoneal macrophages (4) after intravenous administration of *L. monocytogenes*. Urinary NOx was proportional to the amount of orally administered *L. monocytogenes* (Fig. 3) and correlated with the number of viable counts of listeria in MLNs (r = 0.99). Using enteroinvasive and noninvasive pathogens, Witthöft et al. (32) showed that invasion is a prerequisite for activation of the inducible NO synthase. In addition, oral inoculation of a pathogenic *E. coli* strain that colonizes the intestinal tract, but is incapable of translocation, does not result in increased urinary NOx (I. M. J. Bovee-Oudenhoven, unpublished results). Therefore, translocation is a prerequisite for induction of urinary NOx excretion. The observation that heat-killed *L. monocytogenes* is ineffective in stimulating urinary NOx excretion also indicates that translocation is indeed obligatory for induction of the NO response by this pathogen. Thus, urinary NOx is a suitable biomarker for the diagnosis of listeriosis in humans.

In conclusion, a dose-dependent relationship between urinary NOx and translocation of listeria exists in a rat model of oral listeriosis. The kinetics of the urinary NOx response after listeria infection is different from that observed after salmonella infection (6, 27), which increases from day 3 and reaches peak values on days 6 and 7, returning to baseline levels on day 12 after inoculation. This may be explained by the time course of infection. Compared with listeria, salmonella infection is slowly cleared in rats. Whereas viable listeria counts in systemic organs increase to peak values at day 3 of the infection and are completely cleared 7 days after inoculation (24, 28), viable salmonella counts in lymphoid tissue start to increase at day 3 and beyond (10), with considerable amounts still detectable in MLNs at day 7 of infection (27).

It has been shown that NOx is increased in plasma and urine of patients with acute infective gastroenteritis induced by pathogens such as salmonella, shigella, and campylobacter (1, 8, 9, 11). Plasma NOx correlated with the severity of infection (8). Thus, besides application to the quantitative determination of orally acquired listeriosis in animal models, urinary NOx might also be useful to monitor the efficacy of treatment of listeriosis in humans.

In conclusion, a dose-dependent relationship between urinary NOx and translocation of listeria exists in a rat model of orally acquired *L. monocytogenes* infection. Therefore, excretion of NOx in urine can be used as a noninvasive biomarker for quantifying translocation of this pathogen in animal models and may provide a tool to study the efficacy of functional food components. Besides this application, urinary NOx may also be used to monitor the severity of listeriosis in humans.

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