Defining Inoculation Conditions for the Mouse Model of Ascending Urinary Tract Infection that Avoid Immediate Vesicoureteral Reflux yet Produce Renal and Bladder Infection

James R. Johnson and Jennifer J. Brown

A satisfactory mouse model of ascending urinary tract infection (UTI) must avoid inoculation-induced vesicoureteral reflux (VUR) yet still produce kidney and bladder infection in a substantial proportion of mice. To define inoculation conditions that would satisfy both these conditions, mice were evaluated for VUR immediately after inoculation under a variety of conditions and were assessed for kidney and bladder infection 48 h after inoculation. Elimination of VUR required a slowed infusion rate, a reduced inoculum volume (25 μL), and use of less traumatic methods for euthanasia and organ harvest. Bladder and kidney infection were highly prevalent at 48 h among mice inoculated under VUR-free conditions with either of 2 wild type Escherichia coli strains. Together with reports from other investigators, these findings indicate that satisfactory experimental conditions for the mouse model of ascending UTI can be defined empirically but may be laboratory-specific.

The currently used nonobstructive mouse model of ascending urinary tract infection (UTI) has been an important tool for studying the pathogenesis of UTI [1, 2]. It is designed to avoid the nonphysiologic urinary tract manipulations of many previous animal models of UTI [3], in order to better mimic “uncomplicated” UTI in humans, that is, UTI occurring in the absence of underlying anatomic or functional abnormalities of the urinary tract [4].

Unfortunately, the 50-μL inoculum volume that is commonly used in this model induces immediate vesicoureteral reflux (VUR) in some mice [5, 6]. This not only carries the bacterial inoculum directly to the upper urinary tract, thereby bypassing an important step in the pathogenesis of upper UTI (i.e., bacterial ascent up the ureter), but also causes hydrostatic injury to the renal parenchyma, thereby converting the infection from “uncomplicated” to “complicated” [4, 5]. Others have reported success in circumventing this problem by using a Harvard pump for controlled, slow administration of a 50-μL inoculum volume [7] or by manually inoculating a smaller volume (10 μL) into the urethra rather than the bladder [6].

In addition to avoiding nonphysiologic manipulations of the host, a satisfactory animal model of upper UTI must produce the desired end point with sufficient frequency to allow meaningful comparisons. In our previous studies using the mouse model, renal infection occurred in a high proportion of mice challenged with virulent Escherichia coli strains [8, 9]. However, these experiments involved inoculation conditions that induced VUR, calling into question the relevance of the findings. The literature contains conflicting reports regarding the frequency of upper UTI achievable in the mouse UTI model when ostensibly VUR-free inoculation conditions are used [6, 7]. We undertook the present study to determine whether by altering inoculation conditions we could avoid immediate VUR in the mouse model of UTI...
while still obtaining a satisfactory yield, not only of bladder but also of renal infection.

**Materials and Methods**

*Bacterial strains.* E. coli H5R11 is a spontaneous rifampin-resistant mutant of strain H5 (O18ac:K5:H−), a urosepsis isolate that is virulent in the mouse model of UTI [9, 10]. Strain CP9 (O4:K54) is a human sepsis isolate with documented virulence in several animal models of infection [11, 12]. Both strains express P-fimbriae, hemolysin, and type 1 fimbriae, but whereas H5R11 has a functional aerobactin system, CP9 does not (not shown). Strains were stored at −70°C in Luria-Bertani (LB) broth [13] plus glycerol.

*Bacterial suspensions.* Bacteria from frozen stocks were cultured overnight in static (H5R11) or shaking (CP9) LB broth at 37°C, harvested by centrifugation, and adjusted to the desired concentration either in LB broth with an equal volume of autoclaved India ink (VUR experiments; strain H5R11, ~10⁶ cfu/mL) or in PBS (pH 7.4) without India ink (infection experiments).

*Mouse model of ascending UTI.* The mouse model was based on that of Hagberg et al. [1], with modifications [7, 9]. Female Swiss-Webster mice, age 6–8 weeks (weight range, 17–28 g), were anesthetized with pentobarbital intraperitoneally. The peritoneum was disinfected and a polyethylene catheter (0.28 mm internal diameter, 0.61 mm outer diameter, ~25 mm long) was inserted through the urethra into the bladder until resistance was felt, then withdrawn several millimeters. Urine was expelled by gentle suprapubic pressure.

A tuberculin syringe and connecting tubing were filled with the bacterial suspension and fitted into a Harvard pump. The tubing was connected to the urethral catheter, and the desired volume was delivered into the mouse by running the Harvard pump at a preset rate for a specified time (calibration experiments demonstrated reproducible delivery of target volumes, ± ~5%). The inoculum volume for an individual mouse was determined by multiplying the target volume by mouse weight/20 g.

In VUR experiments, mice were sacrificed immediately after inoculation either by cervical dislocation or by pentobarbital overdose. The abdominal fur was disinfected and the skin was separated (either by forceful traction or by sharp dissection) to expose the abdominal wall, which then was opened sharply. Ureters and kidneys were inspected for grossly visible India ink. Swab cultures were taken from each side of the peritoneal cavity around each kidney and plated separately. Kidneys were then sequentially excised for semiquantitative culture.

In infection experiments, mice were allowed to recover from anesthesia after inoculation, then were euthanized by pentobarbital overdose on the second day after inoculation and processed as in VUR experiments. In addition, with the peritoneum open, urine was aspirated from the bladder using a 27-gauge needle, and the bladder was resected for quantitative culture.

Resected kidneys were halved; one-half of each kidney was cultured semiquantitatively on LB agar using a smear technique as described by O’Hanley et al. [14]. A renal infection intensity score was assigned depending on the number of colonies observed (none = 0; 1–10 = 1; 11–20 = 2; 21–999 = 3; ≥1000 but not confluent = 4; confluent lawn = 5). For each mouse, the combined renal infection intensity score (0–10) was the sum of the renal infection intensity scores for each of its two kidneys. Bladders also were hemisected, and one-half was homogenized in LB broth and cultured quantitatively [5]. Bladder bacterial concentrations were analyzed as log₁₀[(bladder cfu/mL) + 1]. Bacterial isolates from peritoneal, kidney, and bladder cultures were assessed phenotypically for identity with the inoculum strain [10]. Mice whose postmortem cultures showed organisms not corresponding with the inoculum strain were excluded from analysis.

*Statistical methods.* Comparisons of proportions were tested using Fisher’s exact test.

**Results**

Inoculation conditions similar to those reported by others to be reflux-free [7] yielded culture-evident VUR in ~50% of mice and kidneys (table 1, group 1). Visible ink in the upper urinary tract was noted bilaterally in one mouse, which also had bilateral positive peritoneal swab cultures.

Neither slowing the inoculation rate (groups 2 and 3) or reducing the inoculum volume to 25 μL (group 3) lowered the frequency of VUR or eliminated positive peritoneal cultures (table 1). Changing to pentobarbital overdose from cervical dislocation as the method of euthanasia to avoid the reflex voiding observed during cervical dislocation, together with increasing the inoculation rate slightly to avoid the pulsatile flow seen at 0.49 μL/s, reduced (but did not eliminate) VUR; however, neither upper urinary tract ink nor a positive peritoneal culture was encountered (table 1, group 4).

To avoid artifactual VUR possibly resulting from the traction method used in groups 1–4 to expose the abdominal wall, sharp dissection was substituted, with inoculation conditions otherwise held constant. With this modification, 20 consecutive mice were inoculated with 25 μL without any evidence of VUR or a single positive peritoneal culture (table 1, group 5). Differences in the frequency of VUR were statistically significant when group 5 was compared with group 1 (table 1) and with groups 1–4 combined (not shown). However, when the inoculum volume was restored to 50 μL (with other aspects of the procedure unchanged), reflux again occurred in an unacceptable proportion of mice and kidneys (table 1, group 6).

To determine whether the reduced inoculum volume (25 μL) that finally gave VUR-free inoculation was sufficient to establish kidney and bladder infection, we inoculated mice with strains H5R11 or CP9 in varying concentrations using inoculation conditions as for group 5 and euthanized them 48 h later. Kidney and bladder infection was identified in most mice given strain H5R11 and all mice given strain CP9 (table 2). The intensity of infection, as reflected by renal infection intensity scores or bladder bacterial concentrations, was substantial (table 2).

**Discussion**

We found that even with slow, controlled delivery of a bacterial suspension with a Harvard pump, we were unable to instill
Table 1. Impact of inoculation conditions on frequency of vesicoureteral reflux.

<table>
<thead>
<tr>
<th>Group (no of mice)</th>
<th>Inoculation</th>
<th>Method of euthanasia</th>
<th>Method of peritoneal exposure</th>
<th>No. with positive peritoneal kidney culture (mice/kidneys)</th>
<th>No. with ink in upper urinary tract (mice/kidneys)</th>
<th>No. with positive peritoneal swab culture (mice/kidneys)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (6)</td>
<td>50 mL 1.27</td>
<td>Dislocation</td>
<td>Blunt</td>
<td>3/5</td>
<td>1/2</td>
<td>2/3</td>
</tr>
<tr>
<td>2 (6)</td>
<td>50 mL 0.77</td>
<td>Dislocation</td>
<td>Blunt</td>
<td>5/9</td>
<td>1/1</td>
<td>0/0</td>
</tr>
<tr>
<td>3 (10)</td>
<td>25 mL 0.49</td>
<td>Dislocation</td>
<td>Blunt</td>
<td>8/14</td>
<td>1/2</td>
<td>1/1</td>
</tr>
<tr>
<td>4 (5)</td>
<td>25 mL 0.65</td>
<td>Overdose</td>
<td>Blunt</td>
<td>2/2</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>5 (20)</td>
<td>25 mL 0.65</td>
<td>Overdose</td>
<td>Sharp</td>
<td>0*/0*</td>
<td>0/0</td>
<td>0*/0*</td>
</tr>
<tr>
<td>6 (5)</td>
<td>50 mL 0.65</td>
<td>Overdose</td>
<td>Sharp</td>
<td>2/3</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

NOTE. Dislocation = cervical dislocation; overdose = overdose of pentobarbital; blunt and sharp = blunt and sharp dissection of abdominal skin.
* P < .01 vs. group 1.
† P < .05 vs. group 1.

Table 2. Infection outcomes at 48 h with a 25-µL inoculum volume.

<table>
<thead>
<tr>
<th>Strain (no. of mice)</th>
<th>Log₁₀ inoculum concentration (cfu/mL)</th>
<th>Proportion of mice with positive kidney culture</th>
<th>Mean no. of culture-positive kidneys/mouse</th>
<th>Mean renal infection intensity score</th>
<th>Proportion of mice with positive bladder culture</th>
<th>Mean log₁₀ bladder bacterial concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5RII (10)</td>
<td>9.4</td>
<td>0.60</td>
<td>1.0</td>
<td>3.1</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>H5RII (12)</td>
<td>10.3</td>
<td>0.83</td>
<td>1.1</td>
<td>3.2</td>
<td>0.67</td>
<td>2.1</td>
</tr>
<tr>
<td>H5RII (11)</td>
<td>11.2</td>
<td>0.91</td>
<td>1.2</td>
<td>3.8</td>
<td>0.91</td>
<td>4.1</td>
</tr>
<tr>
<td>CP9 (5)</td>
<td>9.7</td>
<td>1.00</td>
<td>2.0</td>
<td>5.8</td>
<td>1.00</td>
<td>4.9</td>
</tr>
<tr>
<td>CP9 (9)</td>
<td>11.0</td>
<td>1.00</td>
<td>1.8</td>
<td>5.8</td>
<td>1.00</td>
<td>4.9</td>
</tr>
</tbody>
</table>

NOTE. NA = not applicable (no culture-positive bladders).

a 50-µL volume into the mouse lower urinary tract without causing VUR in a substantial proportion of mice. This is largely consistent with our earlier studies involving manual inoculation, in which VUR occurred even with a 50-µL inoculum volume [5, 8, 9], and with the experience of Hopkins et al. [6], who reported VUR with inoculation of 50 µL (and even 10 µL) into the mouse bladder.

However, our results seemingly conflict with those of Moley et al. [7] and Johnson et al. [15], who in control experiments detected no VUR in mice inoculated with a 50-µL volume. These discrepancies may be due in part to differences in mouse strain, anesthetic method, or mouse size. In addition, these investigators inoculated their VUR control mice with the peritoneum already opened (Johnson D, personal communication). Although permitting direct visualization of the urinary tract during inoculation and avoiding possible laparotomy-induced VUR, this approach also might have allowed more bladder expansion during inoculation than would occur with a closed abdomen, thereby avoiding pressure increases that could cause VUR in intact mice during actual infection experiments.

We were able to avoid VUR with an inoculum volume of 25 µL, provided that mice were euthanized by overdose rather than cervical dislocation and were opened atraumatically after inoculation. In contrast, Hopkins et al. [6] observed VUR even with a 10-µL volume when delivered into the bladder and were able to avoid VUR only by instilling the 10-µL inoculum into the urethra. We suspect that our success in avoiding VUR with a 25-µL inoculum volume may be due to our slower inoculation rate (0.65 µL/s; cf. 2–3 µL/s) and the controlled infusion provided by the Harvard pump. However, our results also suggest that other experimental conditions, possibly including the methods used for euthanasia and abdominal access, may determine whether VUR will occur. It should be noted that Hopkins et al. killed their mice by cervical dislocation, which in our study appeared to contribute to VUR (table 1).

Since we did not attempt to precisely define the position of the catheter tip within the mouse urinary tract, we cannot comment on possible differences between a urethral and a bladder site of inoculation [6]. Our slight withdrawal of the catheter after initial insertion may have retracted the tip into the urethra in some (or all) mice.

For effective study of upper urinary tract infection, a mouse model of ascending UTI not only must avoid VUR during inoculation but also must produce upper UTI with a usably high frequency. Hopkins et al. [6] advocated a 10-µL intraurethral inoculation method because it avoided VUR and produced bladder infection in nearly all mice. However, it also yielded a very low prevalence of kidney infection (7% overall; 17% at 2 days), despite the administration of 10⁸ cfu of a urovirulent wild type E. coli strain [6]. In contrast, with our 25-µL inoculum, we obtained a high frequency of kidney infection with each of 2 different wild type E. coli strains (table 2), both with
urovirulence phenotypes similar to that used by Hopkins et al. [6]. The lowest total inoculum we administered was roughly equivalent to that used by Hopkins et al. This evidence suggests that with the same absolute bacterial load, 25 μL may be more effective than 10 μL for establishing renal infection in the mouse model. An alternative explanation for our higher frequency of renal infection would be unmeasured virulence differences between our strains H5R11 and CP9 and the strain used by Hopkins et al., E. coli 1677, despite the observed phenotypic similarities.

Finally, our results suggest that for each laboratory that wishes to use an atraumatic mouse model for the study of upper UTI, it is probably advisable to determine empirically inoculation conditions that in their hands avoid VUR yet still give a sufficient prevalence of kidney infection to allow studies of pathogenesis. In such determinations, a sensitive method such as organ culture should be used to detect VUR rather than only inspection for dye or ink in the upper urinary tract [5].

Acknowledgments

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References