Protocol
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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Using a zebrafish model to understand adherent-invasive Escherichia coli infection

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SUMMARY

A zebrafish model was developed to study AIEC colonization, invasion, and inflammation. This model can also be used to study the beneficial effects of a probiotic on AIEC infection of adult zebrafish. Bacteria are grown in vitro and then fish are infected with AIEC by immersion. Subsequently, colonization and inflammation can be assessed. Exposing fish to probiotic at different time points relative to AIEC can determine beneficial effects of probiotics as prophylactics or therapeutics against AIEC.

For complete details on the use and execution of this protocol, please refer to Nag et al. (2022).

BEFORE YOU BEGIN

The protocol below describes the specific steps for using zebrafish as a model for AIEC colonization, invasion and inflammation in the zebrafish gut. AIEC, an opportunistic pathogen, is a major causative agent for inflammatory bowel disease, Crohn’s disease, and ulcerative colitis. Zebrafish have been developed for studying several different enteric pathogens. Animal models to study AIEC pathogenesis are very limited. AIEC models have been primarily cell-culture based, such as in murine epithelial cells and macrophages. Here, we describe a zebrafish model to study AIEC infection. This protocol also describes how to test a probiotic therapeutically and prophylactically in combating the disease caused by AIEC. Zebrafish are useful for studying numerous infectious diseases, in particular enteric diseases. Zebrafish is a natural model for Vibrio cholerae infection (Runft et al., 2014; Nag et al., 2018a, 2018b, 2020) and immune responses (Farr et al., 2021, 2022), as well as for other enteric pathogens including Salmonellae (Howlader et al., 2016) and Shigellae (Howlader et al., 2022). Recently a zebrafish model was developed to study AIEC, which is an opportunistic pathogen causing inflammation, sometimes progressing to inflammation-induced colon cancer during unfavorable conditions in the gut (Nag et al., 2022).

Institutional permissions

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Wayne State University (approval number:20-12-3017). This method was first described in Nag et al. (2022).

Preparation of AIEC

© Timing: 2 days
1. Inoculate 5 mL of LB broth with Ampicillin 100 µg/mL (LB-Amp)) with an isolated AIEC colony from an LB plate (streaked from glycerol stock) and incubate at 37°C with shaking (180 rpm) for 6–8 h (Figure 1A).

2. Inoculate 25 mL of sterile LB/Amp in a 250 mL conical flask with 50 µL of the above starter culture. Incubate it for 14–16 h (overnight) at 37°C with shaking (150 rpm) (Figure 1B).

3. Harvest the cells by centrifugation at 6,000 g for 10 min. Remove the supernatant with a pipette or decant into a disinfectant solution. Resuspend the cells in 1 mL sterile PBS to make a concentrated cell suspension (Figure 1C).

4. Pipet 10 mL sterile PBS into a 15 mL tube and gradually add the concentrated cell suspension until the optical density at 600 nm (OD600) of the final suspension equals 1 (Figure 1D). This will put the suspension at ~10^9 CFU/mL.

△ CRITICAL: First, add 250 µL of concentrated cell suspension to 10 mL PBS and check the OD. If the OD is below 1, add a small amount of the concentrated cell suspension in increments (10–20 µL, depending on the last OD value) to increase the effective OD in the final suspension to OD=1. If the OD is greater than 1, add PBS gradually to make the OD=1.

5. Plate serial dilutions of the final suspension to quantify the actual CFU of the inoculated bacteria (Figure 1E).

**Preparation of E. coli Nissle**

⊙ Timing: 2 days
6. Prepare culture by inoculating 5 mL of LB containing Rifampicin 75 μg/mL (LB/Rif)) with an isolated E. coli Nissle colony from an LB plate and incubate at 37°C with shaking (180 rpm) for 6–8 h (Figure 1A).

7. Prepare subculture by inoculating 25 mL of sterile LB/Rif in a 250 mL conical flask with 50 μL of the above starter culture. Incubate for 14–16 h (overnight) at 37°C with shaking (150 rpm) (Figure 1B).

8. Harvest the cells by centrifugation at 6,000 g for 10 min. Remove the supernatant with a pipette or decant into disinfectant solution. Resuspend the cells in 1 mL sterile PBS to make a concentrated cell suspension (Figure 1C).

9. Pipet 10 mL sterile PBS into a 15 mL tube and gradually add the concentrated cell suspension until the optical density at 600 nm (OD600) of the final suspension equals 1 (Figure 1D). This will put the suspension at ~10^9 CFU/mL.

10. Plate serial dilutions of the final suspension to quantify the actual CFU of the inoculated bacteria (Figure 1E).

### Preparation of zebrafish gut for quantification of inflammatory markers

**Timing:** 2 h

11. After euthanizing and dissecting zebrafish (the euthanization protocol is described later), place the whole digestive tract (gut) into a 1.5 mL tube (Figures 2A and 2C).

12. For RNA isolation add 200 μL TRIzol and homogenize the gut with a 1.5 mL tube pestle homogenizer. Add another 800 μL of TRIzol (Figure 2B).

    △ CRITICAL: TRIzol is a hazardous material containing phenol. Use personal protective equipment including, gloves, lab coat and eye protection. Perform the phenol related steps in a fume hood, if possible.

13. For ELISA Add 100 μL of PBS and 50 μL of RIPA buffer to the gut and homogenize the gut with 1.5 mL tube pestle homogenizer (Figure 2D).
To do ELISA from stool, centrifuge the 50 mL excreted water at 10,000 g for 10 min and collect the stool at the bottom by resuspending in 1 mL PBS (Figure 2E).

△ CRITICAL: Harvested zebrafish gut can be used for mounting, section preparation and microscopy studies following the conventional procedure.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                      | SOURCE                                           | IDENTIFIER |
|-----------------------------------------|--------------------------------------------------|------------|
| **Bacterial and virus strains**         |                                                  |            |
| Adherent invasive *E. coli* strain LF 82 | Kind gift from Prof. A. Darfeuille- Michaud     | N/A        |
| Adherent invasive *E. coli* strain LF 82 (Spec 100 μg/mL) | Kind gift from Dr. Olivier Espeli | N/A        |
| *E. coli* strains Nissle 1917           | Lab Strain                                       | N/A        |
| *E. coli* strain Nissle (rif 75 μg/mL)  | Lab Strain                                       | N/A        |
| **Chemicals, peptides, and recombinant proteins** |                                                  |            |
| Tryptone                                | BD                                               | Ref: 211705|
| Yeast Extract                           | BD                                               | Ref: 212750|
| Agar                                    | BD                                               | Ref: 214010|
| Instant Ocean salt                      | Aquarium Systems                                 | Product No. SS15-10 |
| Tricaine-S (Ethyl 3-aminobenzoate methanesulfonate) | Syndel                               | Lot No: 13065|
| TNBS (2,4,6-Trinitrobenzenesulfonic acid solution) | Sigma                               | CAS no. 2508-19-2 |
| Trizol                                  | Thermo Fisher Scientific                         | Ref: 15596026|
| SuperScript™ First-Strand Synthesis System | Invitrogen                              | Cat. no: 18080051|
| SYBR green                              | Applied Biosystems                               | Cat. no: A25742|
| Calprotectin ELISA Kit                  | MyBioSource                                      | MBS760803  |
| **Zebrafish**                           | Lab bred, can also be acquired from ZIRC (http://zebrafish.org) | Lab Strain (others can also be used.) Adult fish 3–9 months of age of either sex. |
| **Oligonucleotides**                    |                                                  |            |
| IL1β                                    | Integrated DNA Technologies                      | F5’CATTTGCAAGGGCTCACAG3’; R5’GGACATGCTGAAGGCTACCTG3’ |
| TNFa                                    | Integrated DNA Technologies                      | F5’CCATGCACTCTTGACATCCTG3’; R5’TGGAGGCGTATTGCAGTCAAG3’ |
| IFNγ                                    | Integrated DNA Technologies                      | F5’CTTCCAGGCAAGGATGCAG3’; R5’TCAGCTCAAAACAGAGCCTCG3’ |
| S100A-10b                               | Integrated DNA Technologies                      | F5’GCAGGGGAAACTCTACCAAC3’; R5’CCACCCGCAAGAGACAAAC3’ |
| b-actin                                 | Integrated DNA Technologies                      | F: 5’TCTGTCTTTTCCCCCTCATG3’; R: 5’TTCTGTCCTCCATGCAACCA3’ |
| **Software and algorithms**             |                                                  |            |
| GraphPad Prism 9.0                      | GraphPad Prism Software, Inc                     | https://www.graphpad.com/ |
| Microsoft Office                        | Microsoft                                        | N/A        |
| **Other**                               |                                                  |            |
| RNase-Free Disposable Pellet Pestles    | Fisherbrand                                      | 12-141-368 |
| NanoDrop™ 2000/2000c Spectrophotometers | Thermo Scientific                                | ND-2000    |

### STEP-BY-STEP METHOD DETAILS

**Determination of AIEC intestinal colonization and invasion in the zebrafish gut**

**Timing:** 3 days

This part of the protocol describes the detailed procedure for zebrafish infection with AIEC and also describes the protocol for assessing the colonization and invasion of AIEC in the zebrafish gut.

1. Inoculation of zebrafish by immersion.
a. Inoculation and incubation (Figure 3A).
   i. Autoclave reverse-osmosis water containing 60 mg/L sea salts to use as the infection water.
   ii. Take 4–5 adult zebrafish (6–12 months old, male or female) from their tank with a fish net.
   iii. Place zebrafish into 400 mL of sterile infection water in a 600 mL beaker.
   iv. Add 2 mL of AIEC suspension (from preparation detailed above) to get the desired infectious dose (typically $5 \times 10^6$ CFU/mL in 400 mL of infection water).
   v. Cover the beaker with a perforated lid to prevent the fish from jumping out; the top of a 200 μL pipet tip box works well for this.
   vi. Label each beaker and place them into a glass-front incubator set at 28°C for the duration of the experiment.

b. Procedure for transitory exposure (Figures 3A and 3B).

   CRITICAL: Transitory exposure to AIEC (typically 6 h) followed by the removal of the external bacteria in the water is useful for more accurate quantification of bacteria excreted by the fish.

   i. After 6 h exposure, pour the beaker water through a fishnet into a container of 10% bleach to kill the AIEC and to collect the fish.
   ii. Place the fish into a sterile 600 mL beaker containing 400 mL sterile infection water. Allow the fish to swim in this clean water for 5 min to remove AIEC from the surface of the fish.
   iii. Repeat the net procedure (step 1.b.i) and place the fish into another 600 mL beaker containing 400 mL sterile infection water. Keep the fish in this beaker for the duration of the experiment (another 18 h) at 28°C.

2. Euthanasia.
   a. At the desired experimental endpoint, take a sample of the infection water (15 mL is usually sufficient) to quantify excreted bacteria and stool markers for inflammation (such as calprotectin; details in later section).
b. Pour the remainder of the water through a fishnet (to collect the fish) into bleach to kill the AIEC.
c. Place the fish into a 250 mL beaker containing 100 mL infection water plus 336 µg/mL of tricaine. Incubate the fish in this solution at room temperature until 30 min after cessation of opercular movement.

△ CRITICAL: The fishnets should be sterilized with a 10% bleach solution, rinsed well, and dried between uses.

3. Dissection.
   a. Preparation of fish.
      i. Wearing gloves, extract a fish out of the tricaine solution with a disposable plastic spoon or small net and place it on the sterile dissecting surface (a wax dissection tray works well for this).
      ii. Position the fish with its ventral side facing upward and pin it to the dissection tray through the lower jaw, with the blunt end of the pin angled away from the body midpoint. Insert another pin just posterior to the anus, also angled away from the body.
   b. Exposure of intestinal tract (Figures 3C and 3D).
      i. Swab the ventral surface of the fish with a lint-free wipe dipped in 70% ethanol.
      ii. Sterilize a scalpel and Vannas scissors by dipping them in 70% ethanol and flaming them in a Bunsen burner.
      iii. Using a sterile scalpel, make a small incision to penetrate the skin and scales of the belly. Be careful not to cut too deeply, as the intestinal tract is located just under the skin.
      iv. Using sterile scissors, cut carefully along the length of the body between the anus and gills, cutting no deeper than skin level. Do not cut through the anus.
      v. Make lateral cuts with the scissors just below the gills to produce flaps.
      vi. Pin the flaps on each side to the dissecting surface, angling the pins away from the body.

Note: The tips of the pins should be sterilized by flaming them with alcohol before use.

c. Removal of intestinal tract (Figure 3D).

△ CRITICAL: The intestinal tract is a pale, very thin tube on top of the other organs and just under the skin of the fish belly.

Sterilize the forceps by dipping in 70% ethanol and flaming to burn off the ethanol. Remove the entire intestinal tract (typically 12–15 mm in length from mouth to anus). Place the intestine into a homogenization tube containing glass beads (see steps 4.a–4.b) and 1 mL of PBS or LB, on ice.

4. Homogenization (Figure 3E).
   a. Prior to beginning the experiment, add ~1.5 g of 1 mm glass beads to 2 mL screw cap tubes (fill them approximately half-way) and sterilize by autoclaving.
   b. Add 1 mL of sterile LB or PBS to the sterile homogenization tubes.
   c. After zebrafish intestines have been added (step 3.c.i) to the tubes, screw the caps on very tightly and secure the tubes in the bead-beating homogenizer.
   d. Homogenize the samples for 1 min on the maximum setting, then cool them on ice for 1–2 min. Repeat this homogenization cycle once more.

△ CRITICAL: Several alternative homogenization methods, such as grinding with a sterile pestle, also work.

5. Quantifying intestinal AIEC colonization levels (Figures 3E and 3G).
   a. For each fish, prepare a series of 5 or 6 1.5 mL microcentrifuge tubes for serial dilution of the intestinal homogenate by adding 900 µL of LB or PBS to each tube.
i. Make 10-fold serial dilutions of the intestinal homogenate by adding 100 μL of homogenate to the first tube.
ii. Vortex it to mix thoroughly.
iii. Add 100 μL from the first tube to the second tube.
iv. Repeat this dilution process until all tubes have been used.
b. Plate 100–200 μL of each dilution on LB agar plates containing 75 μg/mL of Ampicillin (if using Ampicillin resistant strains).
c. Incubate the plates at 37°C for 16–18 h.
d. After the overnight incubation, count AIEC colonies on the plates using an automated colony counter or by manually counting the colonies. In the latter case it is helpful to mark the counted colonies with a marker tip.
e. Determine the CFU per fish intestine by multiplying the CFU on the plate by the dilution factor of the suspension, also considering the volume that was plated.

6. Quantifying intestinal invasion levels.
a. Take the whole intestine from step 3.c in 250 μL sterile PBS and chop it into small segments using sharp scissor tips.
b. Incubate the small intestinal segments with 100 mg/mL of Gentamicin in sterile PBS at room temperature for 25 min to remove the surface colonizing bacteria.
c. Wash intestinal segments three times in sterile PBS for 10 min with periodic mild vortexing.
d. Homogenize the washed intestinal segments with glass beads as described in step 4.
e. Serially diluted and plate the homogenate as described in step 5 for CFU count of the internalized bacteria.

7. Quantifying the bacterial load in excreted water (Figures 3F and 3G).
a. Take a water sample at the desired time point and serially dilute it as described previously.
b. Plate 100 μL of each of the serial dilutions on selective media (LB agar containing the appropriate antibiotic) and incubate at 37°C for 16–18 h.
c. Count the colonies. Calculate the CFU per mL of water using serial dilutions as described in step 5.

**Administration of probiotic strain *E. coli* Nissle to assess its effect on AIEC colonization**

© Timing: 3 days

This part of the protocol describes the detailed procedure for zebrafish inoculation with EcN. It also describes the protocol for testing the effects of EcN on co-inoculation with AIEC, pre-inoculation with AIEC (prophylactically), and post-inoculation with AIEC (therapeutically).

⚠ CRITICAL: The probiotic strain, *E. coli* Nissle, can be administered in three ways, depending on the desired study: by co-inoculation (same time as AIEC infection), prophylactically (before AIEC infection), and therapeutically (after AIEC infection).

8. Inoculation of *E. coli* Nissle with AIEC.
a. Co-Inoculation and incubation.
   i. Place four or five zebrafish into 400 mL of sterile infection water in a 600 mL beaker.
   ii. Add 2 mL of AIEC (cell suspension, described above) and 2 mL of *E. coli* Nissle to 400 mL sterile infection water at the desired infectious dose (typically 5 × 10⁶ CFU/mL for both strains).
   iii. Cover the beaker with a perforated lid to prevent the fish from jumping out; the top of a 200 μL tip box works well for this.
   iv. Label each beaker and place them into a glass-front incubator set at 28°C for 6 h.
   v. After 6 h exposure, pour the beaker water through a fishnet into a container of bleach to kill the AIEC and to collect the fish.
vi. Place the fish into a fresh 600 mL beaker containing 400 mL sterile infection water. Allow the fish to swim in this clean water for 5 min to remove AIEC from the surface of the fish.

vii. Repeat the net procedure (step 8.a.v) and place the fish into another 600 mL beaker containing 400 mL sterile infection water. Keep the fish in this beaker for the duration of the experiment (another 18 h) at 28°C.

b. Prophylactic Inoculation and incubation.
   i. Place four or five zebrafish into 400 mL of sterile infection water in a 600 mL beaker.
   ii. Add 2 mL of *E. coli* Nissle to 400 mL of infection water at the desired infectious dose (typically 5 × 10^5 CFU/mL).
   iii. Cover the beaker with a perforated lid to prevent the fish from jumping out; the top of a 200 µL tip box works well for this.
   iv. Label each beaker and place them into a glass-front incubator set at 28°C for 6 h.
   v. After 6 h of exposure to *E. coli* Nissle, add 2 mL of AIEC and incubate at 28°C for another 6 h.
   vi. After 12 h of incubation, pour the beaker water through a fishnet into a container of bleach to kill the AIEC and to collect the fish.
   vii. Place the fish in a beaker of 400 mL of sterile infection water. Allow the fish to swim in this clean water for 5 min to remove bacteria from the surfaces of the fish.
   viii. Repeat the net procedure (step 8.b.vi) and place the fish into another 600 mL beaker containing 400 mL sterile infection water. Keep the fish in this beaker for the duration of the experiment (another 18 h) at 28°C.

c. Therapeutic Inoculation and incubation.
   i. Place four or five zebrafish into a 600 mL beaker containing 400 mL of sterile infection water.
   ii. Add 2 mL of AIEC to 400 mL of infection water at the desired infectious dose (typically 5 × 10^5 CFU/mL).
   iii. Cover the beaker with a perforated lid to prevent the fish from jumping out; the top of a 200 µL tip box works well for this.
   iv. Label each beaker and place them into a glass-front incubator set at 28°C for 6 h.
   v. After 6 h of exposure, add 2 mL of *E. coli* Nissle to the beaker and incubate at 28°C for another 6 h.
   vi. After 12 h of incubation, pour the beaker water through a fishnet into a container of bleach to kill the AIEC and to collect the fish.
   vii. Place the fish in a beaker of 400 mL of sterile infection water. Allow the fish to swim in this clean water for 5 min to remove bacteria from the surfaces of the fish.
   viii. Repeat the net procedure (step 8.c.vi) and place the fish into another 600 mL beaker containing 400 mL sterile infection water. Keep the fish in this beaker for the duration of the experiment (another 18 h) at 28°C.

9. Euthanization and dissection of zebrafish, and the homogenization and serial dilution of the zebrafish gut can be done as described earlier. Quantifying intestinal colonization and bacterial load in the excreted water can be done for both AIEC and *E. coli* Nissle in their respective antibiotic media following the method previously described.

Quantification of inflammatory markers in the zebrafish gut

⏱ Timing: 1 day

This part of the protocol describes how to do assays such as qRT-PCR and ELISA from zebrafish intestinal samples.

⚠ CRITICAL: Zebrafish specific primary antibodies for most inflammatory markers are not readily available. Therefore, qRT-PCR based quantification of inflammatory markers is performed in most cases.
CRITICAL: Incubate a group of fish for 12 h with 20 μM TNBS (2,4,6-Trinitrobenzenesulfonic acid solution; in infection water to induce non-lethal inflammation as a positive control.

10. qRT-PCR of inflammatory markers.
   a. RNA isolation from whole gut.
      i. Put the whole gut in TRIzol and homogenize using a 1.5 mL tube pestle homogenizer.
      ii. Add 0.2 mL of chloroform, securely cap the tube, then thoroughly mix by shaking and incubate for 2–3 min.
      iii. Centrifuge the sample for 15 min at 12,000 × g at 4°C. The mixture separates into a lower red phenol-chloroform, an interphase, and a colorless upper aqueous phase.
      iv. Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipetting the solution out.
      v. Add 0.5 mL of isopropanol to the aqueous phase, incubate for 10 min at 4°C. Centrifuge for 10 min at 12,000 × g at 4°C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube.
      vi. Discard the supernatant with a micropipettor and resuspend the pellet in 1 mL of 75% ethanol.

Pause point: The RNA can be stored in 75% ethanol for at least 1 year at –20°C, or at least 1 week at 4°C.

   vii. Vortex the sample briefly, then centrifuge for 5 min at 7,500 × g at 4°C. Discard the supernatant with a micropipettor and vacuum or air dry the RNA pellet for 5–10 min.
   viii. Resuspend the pellet in 50–75 μL of RNase-free water.
   ix. If not dissolved completely, Incubate the tube in a water bath or heat block set at 55°C–60°C for 10–15 min.
   x. Use the RNA solution immediately or store it at –80°C.
   b. Measure the RNA concentration using a nanodrop.
   c. Preparation of cDNA from RNA.

Prepare the cDNA from the RNA following the company protocol for SuperScript III First Strand Synthesis System from Invitrogen.
https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FsuperscriptIIIfirststrand_pps.pdf.

d. Sample preparation for qRT-PCR.

CRITICAL: Use PowerUp™ SYBR™ Green Master Mix and Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate with Barcode (Applied Biosystems, Foster City, CA) for the qRT-PCR.

i. Determine the number of reactions you need to set up to see the effect of AIEC induced inflammation. For example, to see the fold change of TNFα (target) during AIEC infection, four groups are needed: cDNA from infected gut with TNFα primers, cDNA from control gut with TNFα primers, cDNA from infected gut with β-actin (housekeeping standard) primers and cDNA from control gut with β-actin primers. Take triplicate for each group. If more than one target genes are there, housekeeping standard can be used once for all the target gene expression.

ii. Make master mix by adding 5 μL of PowerUp™ SYBR™ Green Master Mix, 0.5 μL of forward primer (100 μM), 0.5 μL of reverse primer (100 μM) and 3 μL of nucl ease free water for 1 reaction. Make the master mix for each target gene separately.

CRITICAL: Always make master mix for one extra reaction, which will help during pipetting.
iii. Make a template for the 96 well plate on a paper and mark the wells properly for target genes and sample used.

iv. Add master mix to the corresponding wells according to the template.

v. Then add 1 μL of corresponding cDNA in each well.

vi. Seal the plate with MicroAmp™ Optical Adhesive Film (Applied Biosystems, Foster City, CA).

vii. Mix the solution by mild vortex of the plate and perform a brief centrifuge to move all the solution to the bottom of the wells.

Pause point: The plate can be stored in ice for 5–6 h or at –20°C overnight.

e. qRT-PCR protocol.

i. Perform PCR on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) or any other Real-Time PCR System.

ii. The real-time PCR system was programmed with the following reaction profile: 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by the dissociation curve.

f. Calculation for fold change.

i. Get the threshold cycle number (CT) of triplicate reactions using the ABI-SDS software and determine the mean CT of triplicate reactions.

ii. Calculate ΔCT of the infected (test sample) and control. ΔCT is calculated by the CT of the target gene subtracted from the CT of the housekeeping gene β-actin for the same sample.

iii. Calculate ΔΔCT (or ΔΔCT) by subtracting ΔCT (control) from ΔCT (sample) for a particular target gene.

iv. The levels of expression of the genes of interest were calculated as fold change by the formula 2−ΔΔCT.

v. Make the graph with the fold change value or the log2 fold change value for each gene.

∆ CRITICAL: Each fold change value above 1 is identified as up-regulation of gene expression and each fold change value below 1 is identified as down-regulation of gene expression. For log2 fold change values, all positive values indicate up-regulation and all negative values indicate down-regulation of gene expression.

11. ELISA of inflammatory markers.

a. Add 50 mL of RIPA buffer in 100 μL PBS containing zebrafish gut and homogenize using a 1.5 mL tube pestle homogenizer. Incubate at room temperature for 20 min with periodic vortexing.

b. Resuspend the pelleted stool from excreted water in 1 mL PBS.

c. Dilute the samples (intestinal homogenate or stool suspension) 1:25 with PBS and run the calprotectin ELISA kit according to manufacturer’s instructions (MyBioSource, San Diego, CA).

i. Add nothing to ‘Blank’ well, add 50 μL of standard solution to corresponding standard wells provided by company and add 50 μL of samples to every sample well.

ii. Add 100 μL HRP-conjugated reagent to every well except the blanks.

iii. Cover the plate with aluminum foil (or, the provided cover) and incubate for 60 min at 37°C.

iv. Wash all wells 4 times with 1× wash buffer provided.

v. Add 50 μL of chromogen A and 50 μL chromogen B (light sensitive) to every well. Mix gently and incubate for 15 min at 37°C protecting from light.

vi. Add 50 μL of stop solution to every well.

vii. Read the optical density (O.D.) at 450 nm using an ELISA reader within 5 min (not more than 15 min) after adding stop solution.
viii. Make a standard curve of concentration vs. O.D. using the standard solution readings and make an equation (y=mx+c) to calculate the concentration of unknown samples from their corresponding O.D.

EXPECTED OUTCOMES

The protocol described here uses an adult zebrafish model to study adherent invasive *E. coli* (AIEC) induced colonization, invasion and inflammation. This protocol also describes how to test the effects of a probiotic strain, *E. coli* Nissle (EcN), against AIEC induced infection in zebrafish. This model is useful for studying invasive enteric pathogens.

AIEC is an opportunistic pathogen associated with major inflammatory bowel disease (IBD), Crohn’s disease (CD) and ulcerative colitis (UC). AIEC is a human commensal in some people but during unfavorable gastric conditions it exhibits pathogenic properties and induces inflammation in the gut, sometimes causing inflammation-induced colon cancer (Darfeuille-Michaud et al., 2004). Despite the importance of AIEC to human IBD, animal models to study its pathogenesis are very limited. Instead, models for AIEC have been primarily cell-culture based, such as in murine epithelial cells and macrophages. In this protocol, we established an adult zebrafish model for AIEC induced colonization, invasion, and inflammation. By using the described colonization protocol, we observed AIEC colonization in the zebrafish intestine (Figure 4). We also observed AIEC invasion in zebrafish intestines using the protocol for invasion described above.

EcN is a non-pathogenic Gram-negative strain used as a therapeutic for many gastrointestinal disorders, including diarrhea (Henker et al., 2008), uncomplicated diverticular disease (Fric and Zavoral, 2003) and IBD, particularly UC (Schultz, 2008). In this protocol we describe how to study the effects of EcN on AIEC colonization and invasion. EcN effectively colonized the zebrafish gut (Figure 4) and EcN successfully reduced the colonization of AIEC in zebrafish gut when EcN was co-inoculated with AIEC; used prophylactically and therapeutically.

AIEC can worsen the condition of IBD patients by inducing massive inflammatory responses (Bretin et al., 2018). In the zebrafish model described here, a significant increase in inflammatory markers such as TNFα, IFN-γ and IL-1β during infection was observed (Nag et al., 2022). Calprotectin, a marker for inflammation, is mainly secreted by neutrophils (Jukic et al., 2021). Zebrafish S100A-10b protein is highly similar in protein sequence (43% identity, 67% similarity) to human S100A8, one of two calprotectin subunits (Farr et al., 2022). With this protocol we also observed up regulation of s100a-10b gene expression and elevated S100A-10b protein levels using the human calprotectin ELISA kit during AIEC infection (Nag et al., 2022). EcN treatment reduced the inflammation caused by AIEC with the described protocol (Nag et al., 2022).
QUANTIFICATION AND STATISTICAL ANALYSIS

Each experiment was performed a minimum of three times on separate occasions, unless otherwise specified in the figure legends. Analyzed data are presented as the mean ± standard deviation (SD). Significant frequencies were compared using Student’s t test. A two-tailed t test was performed to test against a control as described in the figure legends. Analyses were performed using GraphPad Prism 7.0.

LIMITATIONS

Although zebrafish showed its potential to study AIEC induced pathogenicity and inflammation, the main limitation was unavailability of a wide variety of primary antibodies and/or ELISA kits to study fish inflammation. Instead, qRT-PCR was used to evaluate changes in the inflammatory markers. The other limitation was getting stool samples of zebrafish to evaluate excreted inflammatory proteins, but this could be resolved by using a more sophisticated set-up (such as, using fine mesh above the container base to collect stool particle) for the zebrafish infection chamber. Most excreted protein studies used excreted water samples, which sometimes diluted the excreted protein to below the level of detection.

TROUBLESHOOTING

Problem 1
Refer to ‘determination of AIEC intestinal colonization and invasion in the zebrafish gut; step 3’.

Gut is difficult to harvest from the fish.

Potential solution
After dissection as described earlier, look for the pale-yellow colored intestine on the top. Move that organ with the forceps tip. The thread-like intestinal structure can be seen. Then take the whole gut from mouth to anus.

Problem 2
Refer to ‘determination of AIEC intestinal colonization and invasion in the zebrafish gut; step 5’.

Sometimes during the colonization assay background colonies from fish intestinal microbiota appear on the antibiotic plates.

Potential solution
Confirm the single colonies on selective media for the bacterial species. Confirm the single colonies with species specific genes, such as, fimH for AIEC.

Problem 3
Refer to ‘preparation of zebrafish gut for quantification of inflammatory markers; step 4’.

Stool is difficult to isolate from the excreted water.

Potential solution
If visible stool pellets are found, harvest the pellets using a 1 mL micropipette with the tips cut (widen the tip by cutting the pointed portion). If the stool is dissolved in water, then take 50 mL and centrifuge it as described earlier putting a mark at the position where the pellet is expected. The pellet of stool might not be visible but rinse the marked area repeatedly with 1 mL PBS to get the concentrated stool suspension.

Problem 4
Refer to ‘determination of AIEC intestinal colonization and invasion in the zebrafish gut; step 2’.
Difficult to determine if the fish is euthanized.

**Potential solution**
The safest way to euthanize fish is by putting them in euthanizing media (water and tricaine) at room temperature or above for at least 30 min after cessation of opercular movement. Euthanasia is assured by organ removal during dissection. Cold water causes a rapid stop in zebrafish movement, which could lead them to appear dead. However, there should still be visible opercular movement in those fish. Therefore it is important to use water at room temperature or higher for euthanasia.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jeffrey Withey (jwithey@med.wayne.edu).

**Materials availability**
This study did not generate new unique reagents. Please contact Prof. Jeffrey Withey (jwithey@med.wayne.edu) to inquire about accessing other materials in this manuscript.

**Data and code availability**
This study did not generate any new code. All data is available from the corresponding author upon reasonable request.

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**AUTHOR CONTRIBUTIONS**
D.N., S.R., and J.H.W. designed the experiments; D.N. and D.F. carried out the experiments; D.N. and J.H.W. wrote the manuscript; and all the authors gave editorial input.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

**REFERENCES**
Bretin, A., Lucas, C., Larabi, A., Dalmaso, G., Billard, E., Barnich, N., Bonnet, R., and Nguyen, H.T.T. (2018). AIEC infection triggers modification of gut microbiota composition in genetically predisposed mice, contributing to intestinal inflammation. Sci. Rep. 8, 12301.

Darfeuille-Michaud, A., Boudeau, J., Bulois, P., Neut, C., Glasser, A.L., Barnich, N., Bringer, M.A., Swidsinski, A., Beaugerie, L., and Colombel, J.F. (2004). High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn’s disease. Gastroenterology 127, 412–421. https://doi.org/10.1053/j.gastro.2004.04.061.

Farr, D., Nag, D., Chazin, W.J., Harrison, S., Thummel, R., Luo, X., Raychaudhuri, S., and Withey, J.H. (2022). Neutrophil-associated responses to Vibrio cholerae infection in a natural host model. Infect. Immun. 90, e0046621. https://doi.org/10.1128/iai.00466-21.

Farr, D.A., Nag, D., and Withey, J.H. (2021). Characterization of the immune response to Vibrio cholerae infection in a natural host model. Front. Cell. Infect. Microbiol. 11, 722520. https://doi.org/10.3389/fcimb.2021.722520.

Fric, P., and Zavoral, M. (2003). The effect of non-pathogenic Escherichia coli in symptomatic uncomplicated diverticular disease of the colon. Eur. J. Gastroenterol. Hepatol. 15, 313–315.

Henker, J., Laass, M.W., Blokhin, B.M., Maydannik, V.G., Bolbot, Y.K., Elze, M., Wolff, C., Schreiner, A., and Schulze, J. (2008). Probiotic Escherichia coli Nissle 1917 versus placebo for treating diarrhea of greater than 4 days duration in infants and toddlers. Pediatr. Infect. Dis. J. 27, 494–499.

Howlader, D.R., Bhaumik, U., Halder, P., Satpathy, A., Sarkar, S., Ghoshal, M., Maiti, S., Withey, J.H., Mitobe, J., Dutta, S., and Koley, H. (2022). An experimental adult zebrafish model for Shigella pathogenesis, transmission, and vaccine efficacy studies. Microbiol. Spectr. 10, e0034722. https://doi.org/10.1128/spectrum.00347-22.

Howlader, D.R., Sinha, R., Nag, D., Majumder, N., Mukherjee, P., Bhaumik, U., Maiti, S., Withey, J.H., and Koley, H. (2016). Zebrafish as a novel model for...
non-typhoidal Salmonella pathogenesis, transmission and vaccine efficacy. Vaccine 34, 5099–5106. https://doi.org/10.1016/j.vaccine.2016.08.077.

Jukic, A., Bakiri, L., Wagner, E.F., Tilg, H., and Adolph, T.E. (2021). Calprotectin: from biomarker to biological function. Gut 70, 1978–1988.

Nag, D., Breen, P., Raychaudhuri, S., and Withey, J.H. (2018a). Glucose metabolism by Escherichia coli inhibits Vibrio cholerae intestinal colonization of zebrafish. Infect. Immun. 86, 00466-18. https://doi.org/10.1128/IAI.00466-18.

Nag, D., Farr, D., Raychaudhuri, S., and Withey, J.H. (2020). An adult zebrafish model for adherent-invasive Escherichia coli indicates protection from AIEC infection by probiotic E. coli Nissle. iScience 25, 104572. https://doi.org/10.1016/j.isci.2022.104572.

Nag, D., Farr, D.A., Walton, M.G., and Withey, J.H. (2020). Zebrafish models for pathogenic vibrios. J. Bacteriol. 202, 001655-20. https://doi.org/10.1128/JB.00165-20.

Nag, D., Mitchell, K., Breen, P., and Withey, J.H. (2018b). Quantifying Vibrio cholerae colonization and diarrhea in the adult zebrafish model. J. Vis. Exp. 57767. https://doi.org/10.3791/57767.

Runft, D.L., Mitchell, K.C., Abuaita, B.H., Allen, J.F., Bajer, S., Ginsburg, K., Neely, M.N., and Withey, J.H. (2014). Zebrafish as a natural host model for Vibrio cholerae colonization and transmission. Appl. Environ. Microbiol. 80, 1710–1717. https://doi.org/10.1128/AEM.03580-13.

Schultz, M. (2008). Clinical use of E. coli Nissle 1917 in inflammatory bowel disease. Inflamm. Bowel Dis. 14, 1012–1018.