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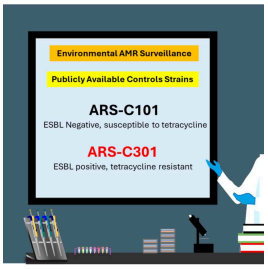
Quantitative Controls for Environmental and Agricultural Surveillance of Extended-Spectrum Beta-Lactamase Producing *Escherichia coli*



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Lisa M. Durso[1], Justine Condon[1], James E. Wells[2], Jonathan G Frye[3], Manan Sharma[4], Abasiofiok M. Ibekwe[5]

[1]USDA Agricultural Research Service (ARS), Agroecosystem Management Research, Lincoln, NE;
[2]USDA Agricultural Research Service (ARS), U.S. Meat Animal Research Center, Clay Center, NE;
[3]USDA Agricultural Research Service (ARS), U.S. National Poultry Research Center, Poultry Microbiological Safety and Processing Research Unit, Athens, GA;
[4]USDA Agricultural Research Service (ARS) Environmental Microbial and Food Safety Laboratory, Beltsville, MD;
[5]USDA Agricultural Research Service (ARS), Agricultural Water Efficiency and Salinity Research Unit, Riverside CA

Lisa M. Durso
USDA - ARS

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We use this protocol and it's working

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Abstract

The importance of standardized control strains for environmental antibiotic resistance (AR) efforts cannot be underestimated. Control strains are useful in standardizing the quality of testing across laboratories and are essential for quality control of experimental procedures and reagents. This protocol introduces two new agriculturally sourced *Escherichia coli* strains for use as environmental AR control strains in method development, surveillance, and research efforts. Both strains are fully characterized, and whole-sequenced, and are available through multiple public sources. ARS-C101 is a control strain that is extended-spectrum β-lactamase (ESBL) negative and tetracycline susceptible. ARS-C301 is a control strain that is ESBL positive, and tetracycline resistant. They are both compatible with the World Health Organization efforts: "WHO integrated global surveillance on ESBL-producing *E. coli* using a “One Health” approach: implementation and opportunities” (aka "Tricycle”). The current protocol describes how to make quantitative controls of ARS-C101 and ARS-C301 control strains using a modification of the WHO Tricycle protocol.

Guidelines

Scope and Application

This protocol describes a method for the creation of quantitative controls, as developed and described by the World Health Organization, with minor modifications, as applied to two publicly available control strains.

World Health Organization, 2021. "WHO integrated global surveillance on ESBL-producing *E. coli* using a "One Health" approach: implementation and opportunities." License: CC BY-NC-SA 3.0 IGO. ISBN: 978-92-4-002140-2 https://www.who.int/publications-detail-redirect/9789240021402

The original protocol for the steps described here can be found in ***section 5.3.6.8 of the WHO Tricycle protocol "Preparation of a quantitative positive control".***

- This protocol describes the creation of quantitative controls from two *Escherichia coli* strains specifically identified for use as controls in the environmental and agricultural surveillance of both extended-spectrum β-lactamase producing and/or tetracycline resistant *E. coli*.

- *E. coli* ARS-C101 is extended-spectrum β-lactamase negative and tetracycline susceptible, and *E. coli* ARS-C301 is extended-spectrum β-lactamase positive and tetracycline resistant.

- Both strains are sequenced **[JF1]** **[LD2]**, and available from the USDA-ARS culture collection and ATCC. The ARS-C301 strain is also available as a pre-made quantitative pellet, from Microbiologics. See Materials section for further details on how to obtain the strains.

This method is being developed as part of a United States Department of Agriculture, Agricultural Research Service National Program 212 project "Managing Manure as a Soil Resource for Improved Biosecurity, Nutrient Availability, and Soil Sustainability".

The mention of firm names or trade products does not imply that they are endorsed or recommended by the USDA or collaborating agencies over other firms or products not mentioned.

Materials

Equipment

1. Incubator maintained at 35°C -37°C
2. Autoclave
3. Pipettors, 1-1000 µL
4. Vortex mixer for dilutions
5. Bunsen Burner
6. Camera (optional for documentation)
7. Water bath maintained at 50°C for tempering agar
8. Refrigerator
9. Ultralow Freezer (preferably -80°C).
10. Quanti-Tray Sealer (89-10894-04, IDEXX, Westbrook, ME)
11. Quanti-Tray Comparator (WQT2KC, IDEXX, Westbrook, ME)

Supplies

12. Gloves
13. Pipette tips P1000, P200, P10
14. Ethanol
15. Sterile graduated cylinders, 100 mL
16. Petri dishes 15 × 100 mm (standard size), for re-streaks
17. Microcentrifuge tubes
18. Sterile loops
19. Sharpie Markers
20. Ice bucket or insulated cooler for holding dilution tubes and samples when plating.
21. Freezer glycerol storage tubes
22. Quanti-Tray/2000 Trays (WQT2KPK, IDEXX, Westbrook, ME)
23. Quanti-Tray bottles, 120 ml (98-09222-00, IDEXX, Westbrook, ME)
24. Colilert snap-packs (98-12973-00, IDEXX, Westbrook, ME)

Media and Reagents

25. Cefotaxime 4 mg/mL Stock

Note

To make cefotaxime 4 mg/mL stock: 0.04 g cefotaxime sodium salt in 10 mL of molecular grade water - filter sterilize.

26. Tetracycline 32 mg/mL Stock

Note

To make 32 mg/mL stock: 32 mg tetracycline in 10 mL of ethanol - filter sterilize.

27. Buffered Peptone Water (BPW) or Phosphate Buffered Saline (PBS)
28. Tryptone Bile X-glucuronide (TBX) Agar (TBX-plain)
29. TBX agar supplemented w/ CTX at 4 ug/mL (TBX-CTX4) (ESBL-E. coli medium)

Note

To make TBX-CTX4**:** For 1 L of TBX media, **after autoclaving and cooling to** 50 °C, Add 1000 µL of CTX stock (4mg/mL) solution.

30. TBX agar supplemented w/ TET at 32 ug/mL (TBX-Tet32)

Note

To make TBX-TET32: For 1 L of TBX media, **after autoclaving and cooling to** 50 °C, Add 1000 µL of TET (32 mg/mL) stock solution

31. Tryptic Soy Agar (TSA)
32. Tryptic Soy Broth (TSB)
33. Glycerol, 30%, for freezer storage

Control Strains

34. ***Escherichia coli* ARS-C101**
    (negative control for cefotaxime and tetracycline resistance, and ESBL production)
35. ***Escherichia coli* ARS-C301**
    (positive control for cefotaxime and tetracycline resistance, ESBL production, and indole test)

ARS-C101 (negative control):

- NCBI accession number PRJNA1003888, BioSample B-65681
- ARS Culture Collection (Accession number: B-65681) **https://nrrl.ncaur.usda.gov/**
- ATCC (isolate ID BAA-3340)

ARS-C301 (positive control):

- NCBI accession number PRJNA1003888, BioSample SAMN36910816

- ARS Culture Collection (Accession number: B-65682) **https://nrrl.ncaur.usda.gov/**
- ATCC (isolate ID BAA-3341)
- Also available through Microbiologics (as a quantitative pellet containing 10-100 CFU) via a custom order (contact industrial microbiology QC products regional sales manager by email requesting a quote for ARS-C301 https://www.microbiologics.com/contact-us)

Troubleshooting

Safety warnings

Safety

Safety information

1. The laboratory protocols described in this document involve culturing antibiotic resistant organisms and working with control strains that are known antibiotic resistant organisms. Procedures should only be performed in approved Biosafety Level 2 (BSL2) laboratories, following all BSL2-safety protocols.

2. Extreme caution is required when performing this procedure, so as not to spill or generate aerosols.

3. All work with the ARS-C301 positive control strain must be performed in a certified BSL2-approved biological safety cabinet.

Quantitative Controls for Environmental and Agricultural Surveillance of Extended-Spectrum Beta-Lactamase Producing *Escherichia coli*

1

Note

The following protocol is taken from section 5.3.6.8 of the WHO Tricycle protocol ***"Preparation of a quantitative positive control"***, with minor modifications.

World Health Organization, 2021. "WHO integrated global surveillance on ESBL-producing *E. coli* using a "One Health" approach: implementation and opportunities." License: CC BY-NC-SA 3.0 IGO. ISBN: 978-92-4-002140-2 https://www.who.int/publications-detail-redirect/9789240021402

Note

Verbatim steps are indicated by "*italic print in quotation marks*"**.**

Citation

World Health Organization (2021)
. WHO integrated global surveillance on ESBL-producing E. coli using a "One Health" approach: implementation and opportunities Licence: CC BY-NC-SA 3.0 IGO.

https://www.who.int/publications/i/item/9789240021402 LINK

Note

***Escherichia coli* ARS-C101 (negative control)** is available from:
- the ARS Culture Collection **https://nrrl.ncaur.usda.gov/** (Accession number: B-65681);
- ATCC (isolate ID BAA-3340).

***Escherichia coli* ARS-C301 (positive control)** is available from:
- the ARS Culture Collection **https://nrrl.ncaur.usda.gov/** (Isolate ID: B-65682);
- from ATCC (isolate ID BAA-3341);
- and as a pre-quantified pellet (10-100 CFU) from Microbiologics (St. Cloud, MN) via a custom order (contact industrial microbiology QC products regional sales manager **https://www.microbiologics.com/contact-us** by email requesting a quote for ARS-C301).

Safety information

Safety

The laboratory protocols described in this document involve culturing antibiotic resistant organisms and working with control strains that are known antibiotic resistant organisms. Procedures should only be performed in approved Biosafety Level 2 (BSL2) laboratories, following all BSL2-safety protocols.

All work with the ARS-C301 positive control strain must be done using a biosafety cabinet.

Quantitative Control - Prepare Cultures - Day 1

2 Inoculate ARS-C101 and ARS-C301 onto individual TSA plates, streaking for isolation. Incubate at 37 °C Overnight . 15m

3 Dilute 40 mL glycerol with 60 mL deionized water, and autoclave.

Quantitative Control - Prepare Cultures - Day 2

4 Wait until the end of the workday, and then pick an isolated colony of each control strain into 9 mL of growth medium (TSB). Incubate at 37 °C for 18:00:00 .

"After one night of growth at 37 °C *, there are approximately 1 X 10^9 CFU per ml in the broth, and the broth should appear visibly turbid" **

Note

*italicized text in quotes is taken directly from https://www.who.int/publications-detail-redirect/9789240021402

Quantitative Control – Prepare and Quantify Dilutions - Day 3

5 *"Prepare 10-fold serial dilutions: Take* 1 mL *of the overnight culture into* 9 mL *diluent and vortex to get a 10^{-1}dilution. Repeat 4 times, yielding 10^{-2}, $10^{-3}$and 10^{-4} dilutions."*

6 *"Add* 0.3 mL *of the 10^{-4} dilution to* 29.7 mL *sterile 40% glycerol and mix well. (This is a further dilution of 100X and thus a 10^{-6} dilution). Incubate for* 00:10:00 *at* Room temperature *and mix again before proceeding."*

7 *"Add* 0.1 mL *of dilution 10^{-6} that was prepared in step 6 to* 0.9 mL *sterile 40% glycerol and mix very well. (This is a 10^{-7} dilution)."*

8 Enumerate the 10^{-4} dilution, 10^{-6} dilution, and 10^{-7}dilution.

Note

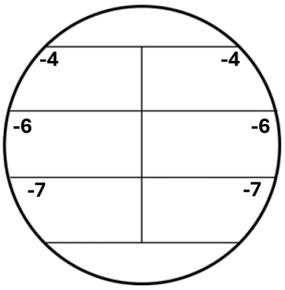
Note: Instead of standard plate dilutions, the method evaluation for the current protocol used Single-Plate Serial Dilution Spotting technique (Thomas et al., 2015). See citation below and this video link: **SP-SDS: A Simple technique for microbial CFU enumeration - YouTube**.

Citation

Thomas, Pious, Aparna C. Sekhar, Rshmi Upreti, Mohammad M. Mujawar, and Sadiq S. Pasha.
(2015)
. Optimization of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured method for bacterial and yeast cfu enumeration and single colony isolation from diverse samples.
Biotechnology Reports 8 :45-55.

https://doi.org/10.1016/j.btre.2015.08.003 LINK

Spot 20 µL each of the 10^{-4}, 10^{-6} , and 10^{-7}dilutions onto the appropriate section of the enumeration plate.



Single Plate Serial Dilution Spotting Template - resize to fit bottom of standard petri plate.

- CTX pos control (ARS-C301) – spot on TBX-CTX4.
- TET pos control (ARS-C301) – spot on TBX-TET32.
- Neg control (ARS-C101) – spot on TBX-Plain.

9 Incubate all plates at 37 °C Overnight .

10 Store the 10^{-4} dilutions (one in diluent, one in glycerol), as well as the 10^{-6}, and 10^{-7} glycerol dilutions in the refrigerator Overnight .

Quantitative Control – Count & Adjust - Day 4

11 Count the colonies in each plate section and calculate the CFU per 100 µL.

12 If desired, take photos of the plates for laboratory notebook.

13 Make freezer stock aliquots of controls containing 100-120 colonies per 100 µL.

13.1 *"If the 10^{-6} dilution contained **more** than 120 colonies/100 µl, look at counts from the 10^{-7} dilution.*
If the 10^{-7} dilution contained 10-100 colonies, dilute the 10^{-6} dilution with sterile 40% glycerol to reach 100 colonies per 100 µL."

- *"For example, if the 10^{-7} dilution contained 80 colonies, dilute the 10^{-6} dilutions 8X (5 ml 10^{-6} dilution + 35 ml sterile 40% glycerol) to achieve about 100 colonies per 100 µL."*

13.2 *"If the 10^{-6} dilution contained **less** than 100 colonies, use the 10^{-4} dilution to prepare an appropriate stock containing 80-100 colonies per 100 µL using sterile 40% glycerol."*

- *"For example, if the 10^{-6} dilution contained 50 colonies, dilute the 10^{-4} dilution 50x (1 mL 10^{-4} dilution + 49 mL sterile 40% glycerol."*

14 Once dilutions containing **100-120 colonies per 100 µL** are obtained, prepare 500 µL aliquots in cryogenic tubes for colony storage. Mix well, incubate for 00:10:00 at Room temperature, and mix again before freezing at -80 °C.

Quantitative Control – Check Counts - Day 5

15 *"Select four random tubes from each control strain, and thaw."*

16 For each control strain, test all four tubes:

- **Step Case 1: via Quanti-Tray**
- **Step Case 2: via Membrane Filtration or Standard Plate Count**

STEP CASE

via Quanti-Tray 4 steps

Enumeration using IDEXX Quanti-Tray/2000®, an MPN-based standard method.

17 Perform Colilert Quanti-Tray/2000 assay, in duplicate, according to the standard protocol using 100 µL of the enumerated *E. coli* stock for each tray.

1) Colilert plain
2) Colilert-CTX4
3) Colilert-TET32

> Note
>
> For Colilert-CTX4, remove cap and add 100 µL of the [M] 4 mg/mL cefotaxime stock solution to 99.9 mL of sterile water. Recap and shake prior to adding to the Quanti-Tray tray. For Colilert-TET32 add 100 µL of the [M] 32 mg/mL tetracycline stock solution to 99.9 mL of sterile water. Recap and shake. For the negative control add 100 mL of sterile water. Recap and shake prior to adding to the Quanti-Tray tray.

- **ARS-C101** (Neg control) – run using Colilert-Plain, Colilert-CTX4, Colilert-TET32.
- **ARS-C301** (CTX & TET pos control) – run using Colilert-Plain, Colilert-CTX4, Colilert-TET32

18 Incubate all trays, cell-side-up, at 35 °C +/-2°C for 24:00:00 1d

19 Determine the counts of the quantitative controls. Counts need to lie within the range of the mean +/- 2x standard deviation.

- *"If out of range, but within 3x standard deviations, investigate whether there was a reason for the deviating counts (i.e. control tubes stored for long times, and counts started to decline in earlier measurements."*
- *"If counts are not within 3x standard deviation of the mean, all measurements made that day need to be rejected."*

20 If desired, take photos of the Quanti-Tray trays for laboratory notebook.

Protocol references

Thomas, P., Sekhar, A.C., Upreti, R., Mujawar, M.M. and Pasha, S.S., 2015. Optimization of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured method for bacterial and yeast cfu enumeration and single colony isolation from diverse samples. *Biotechnology Reports*, *8*, pp.45-55. https://doi.org/10.1016/j.btre.2015.08.003

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Note

Steps in the protocol that contained a citation are listed below.

Citations

Step 1

World Health Organization. WHO integrated global surveillance on ESBL-producing E. coli using a "One Health" approach: implementation and opportunities Licence: CC BY-NC-SA 3.0 IGO.
https://www.who.int/publications/i/item/9789240021402

Step 8

Thomas, Pious, Aparna C. Sekhar, Rshmi Upreti, Mohammad M. Mujawar, and Sadiq S. Pasha.. Optimization of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured method for bacterial and yeast cfu enumeration and single colony isolation from diverse samples
https://doi.org/10.1016/j.btre.2015.08.003

Acknowledgements

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