May 08, 2021

Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces

Rosine Manishimwe[1], Paola M. Moncada[1], Marie Bugarel[1], H. Morgan Scott[2], Guy H. Loneragan[1]
[1]Texas Tech University; [2]Texas A&M University - College Station

1 Works for me dx.doi.org/10.17504/protocols.io.brjkm4kw

Rosine Manishimwe
Texas Tech University

ABSTRACT

This protocol was designed to help researchers with limited laboratory resources to generate valuable information on the status of antibiotic resistance among indicator *E.coli* and pathogenic *Salmonella* from food producing animals.
The protocol uses less technology intensive methods such the disk diffusion and the combination disk test to estimate isolate- and sample-level prevalence of *E.coli* and *Salmonella* resistance to most critical important antibiotics.
In this protocol, agar-based media without antibiotic-supplements are used to isolate *E. coli* and *Salmonella* from fecal samples. Besides, agar-based media supplemented with antibiotics are used to screen for *E. coli* and *Salmonella* resistant to third-generation cephalosporins and to screen for *E. coli* and *Salmonella* with low susceptibility to quinolones. Furthermore, all bacterial isolates are tested for their susceptibility to a panel of twelve antibiotic disks using the disk diffusion. Finally, the combination disk test is used to test for phenotypic production of extended spectrum beta-lactamases (ESBLs) or AmpC among all bacterial isolates resistant to third-generation cephalosporins using a second panel of 12 antibiotic disks.

EXTERNAL LINK
https://doi.org/10.1371/journal.pone.0242390

DOI
dx.doi.org/10.17504/protocols.io.brjkm4kw

EXTERNAL LINK
https://doi.org/10.1371/journal.pone.0242390

PROTOCOL CITATION
Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan 2021. Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. **protocols.io** https://dx.doi.org/10.17504/protocols.io.brjkm4kw

CREATED
Jan 18, 2021

LAST MODIFIED
May 08, 2021

PROTOCOL INTEGER ID
46412

GUIDELINES

- Follow the manufacturer guidelines for media preparation

- Follow the CLSI guidelines for preparation of media supplemented with antibiotics
- Keep all the cultured media until you have obtained all the results

Media preparation.pdf

MATERIALS TEXT

Materials.pdf

DAY 1 : Preparation of fecal samples 2m

1 For each fecal sample
Use a wooden scoopula to weight **10 g** of feces

2 Transfer the feces in a 710mL Whirl Pak® bag (Whirl-Pak, Madison, Wisconsin) with a filter

3 Add **90 mL** of Buffered Peptone Water **Becton-Dickinson** to the bag

4 Stomach at 230 rpm for **00:02:00** 2m

5 Put bags in a container and incubate at **42 °C** **Overnight**

Overnight non-selective enrichments

DAY 2 : Isolation of *E. coli*

6 Remove the bags from the incubator

7 Use a **10 µl** loop to streak the non-selective enrichments onto MacConkey agar **Hardy Diagnostics** plates with no antibiotic supplements to isolate **non-type-specific *E. coli*.**

8 Use another **10 µl** loop to streak the non-selective enrichments onto MacConkey agar **Hardy Diagnostics** plates supplemented with **1.0 mg/L** of Cefotaxime **Acros Organics** to screen for ***E. coli* resistant to third-generation cephalosporins.**

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

9 Use another **10 µl** loop to streak the non-selective enrichments onto MacConkey agar **Hardy Diagnostics** plates supplemented with **0.5 mg/L** of Ciprofloxacine **Acros Organics** to screen for ***E. coli* not susceptible to quinolones.**

10 Incubate all agar-plates at **37 °C** **Overnight**

Bacterial colonies with *E.coli* typical morphology

DAY 2 : Isolation of *Salmonella*

11 For each of the overnight non-selective enrichment
Pipet **1 mL**

12 Transfer it into **9 mL** of Rappaport-Vassiliadis Salmonella broth **Hardy Diagnostics** in a culture tube and vortex mix

13 For each of the overnight non-selective enrichment
Pipet another **1 mL**

14 Transfer it into **9 mL** of Tetrathionate broth **Hardy Diagnostics** in a culture tube and vortex mix

15 Incubate all culture tubes at **42 °C** **Overnight**

Selective Enrichment for *Salmonella*

DAY 3 : Isolation of *E.coli*

16 Remove all MacConkey agar-plates from the incubator

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

17 On each MacConkey agar-plate : Inspected agar-plates to identify growth of bacterial colonies with typical morphology of *E. coli* : pink, convex, circular and dry colonies with a surrounding pink zone.

18 From each type of MacConkey agar-plate (with and without antibiotics), select one isolated *E.coli* typical colony

19 Use a **1 µl** loop to re-streak the colony onto a similar MacConkey agar-plate type

20 Incubate all the agar-plates at **37 °C** **Overnight**

Isolated and pure colonies of *E.coli*

DAY 3 : Isolation of *Salmonella*

21 Remove *Salmonella* selective enrichments from the incubator

22 For each of the enriched selective broths:

- Use a **10 µl** loop to streak the enriched selective broth onto Brilliant Green Sulfa agar **Becton-Dickinson** (BGS) to isolate *Salmonella*
- Use another **10 µl** loop to streak the enriched selective broth onto Xylose Lysine Deoxycholate agar **Hardy Diagnostics** (XLD) to isolate *Salmonella*

23 For each of the enriched selective broths:

- Use another loop to streak **10 µl** of the selective broth onto Brilliant Green Sulfa agar **Becton-Dickinson** supplemented with **1.0 mg/L** of Cefotaxime **Acros Organics** to screen for *Salmonella* resistant to third-generation cephalosporins
- Use another loop to streak **10 µl** of the selective broth onto Xylose Lysine Deoxycholate agar **Hardy Diagnostics** supplemented with **1.0 mg/L** of Cefotaxime **Acros Organics** to screen for *Salmonella* resistant to third-generation cephalosporins

24 For each of the enriched selective broths:

- Use another loop to streak **10 µl** of the selective broth onto Brilliant Green Sulfa agar **Becton-Dickinson** supplemented with **0.5 mg/L** of Ciprofloxacine **Acros Organics** to screen for *Salmonella* not susceptible to

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

quinolones

- Use another loop to streak **10 µl** of the selective broth onto

Xylose Lysine Deoxycholate agar **Hardy Diagnostics** supplemented with **0.5 mg/m**L of

Ciprofloxacine **Acros Organics** to screen for *Salmonella* not susceptible to quinolones

25 Incubate all the inoculated plates at **37 °C** **Overnight**

Bacterial colonies with *Salmonella typical* morphology

DAY 4 : Isolation of *E.coli*

26 Remove all MacConkey plates from the incubator

27 From each of the agar-plates, pick one *E.coli* typical colony

Transfer the colony on a filter paper

28

- Drop 1 or 2 droplet of Indole spot test **Hardy Diagnostics** on the bacterial colony
- If the colony turns blue : the colony is *E. coli* (indole positive)

***E.coli* colonies confirmed**

29 For each of the MacConkey agar-plates with confirmed *E.coli* colonies

- Use a **1 µl** loop to transfer one well isolated colony into **9 mL** of Tryptic Soy Broth **Hardy Diagnostics** in a culture tube
- Vortex mix

30 Incubate all the inoculated culture tubes at **37 °C** **Overnight**

DAY 4 : Isolation of *Salmonella*

31 Remove all *Salmonella* selective agar-plates from the incubator

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

32 Inspected agar-plates to identify growth of bacterial colonies with typical morphology of *Salmonella* :

- On Brilliant Green Sulfa: pink, circular, dry, convex colonies
- On Xylose Lysine Deoxycholate : black, circular convex colonies

33 From each type of *Salmonella* selective agar-plates, select one isolated *Salmonella* typical colony

Use a **1 µl** loop to re-streak the colony onto a similar agar-plate type

34 Incubate all the inoculated plates at **37 °C** **Overnight**

Isolated and pure colonies of *Salmonella*

DAY 5 : Isolation of *E.coli*

35 Remove inoculated culture tubes (with *E.coli* in tryptic soy broth) from the incubator

36 -For each of the *E.coli* culture

- Use a pipettor to transfer **850 µl** of *E. coli* culture into a microcentrifuge tube containing **150 µl** of glycerol

- Vortex mix

37 Keep the isolates at **-20 °C** or **-80 °C** until further processing

DAY 5 : Isolation of *Salmonella*

38 Remove all *Salmonella* selective agar-plates from the incubator

39 For each of the plates:

- Use a **1 µl** loop to pick one isolated colony
- Stab streak the colony onto lysine Iron Agar **Hardy Diagnostics** slants in culture tubes for biochemical testing

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

Don't discard the agar-plates, keep them at 4 °C

40 Incubate all inoculated Lysine Iron Agar slants at **37 °C** **Overnight**

DAY 6 : Isolation of *Salmonella*

41 Remove culture tubes with Lysine Iron Agar slants from the incubator

42 Inspect the slants for typical *Salmonella* reactions:
- Notice a bacterial growth on the slant

- The slant and butt are purple
- The butt blackening with H_2S positive

43 For each of the *Salmonella* selective agar-plates with confirmed *Salmonella* colonies (Positive Lysine Iron Agar):

- Use a **1 µl** loop to transfer one well isolated colony into **9 mL** of Tryptic Soy Broth **Hardy Diagnostics** in a culture tube
- Vortex mix

44 Incubate all the inoculated culture tubes at **37 °C** **Overnight**

DAY 7 : Isolation of *Salmonella*

45 Remove inoculated culture tubes (with *Salmonella* in tryptic soy broth) from the incubator

46 For each of the *Salmonella* culture :
- use a pipettor to transfer **850 µl** of the culture into a microcentrifuge tube containing **150 µl** of glycerol

- Vortex mix

47 Keep the isolates at **-20 °C** or **-80 °C** until further processing

DAY 1: Antibiotic susceptibility testing

48 Remove bacterial isolates to be tested from the freezer

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

49 Use a **10 µl** loop to streak the bacteria isolate on 5% sheep blood agar plates

50 Incubate the all inoculated blood agar-plates at **37 °C** **Overnight**

DAY 2: Antibiotic susceptibility testing

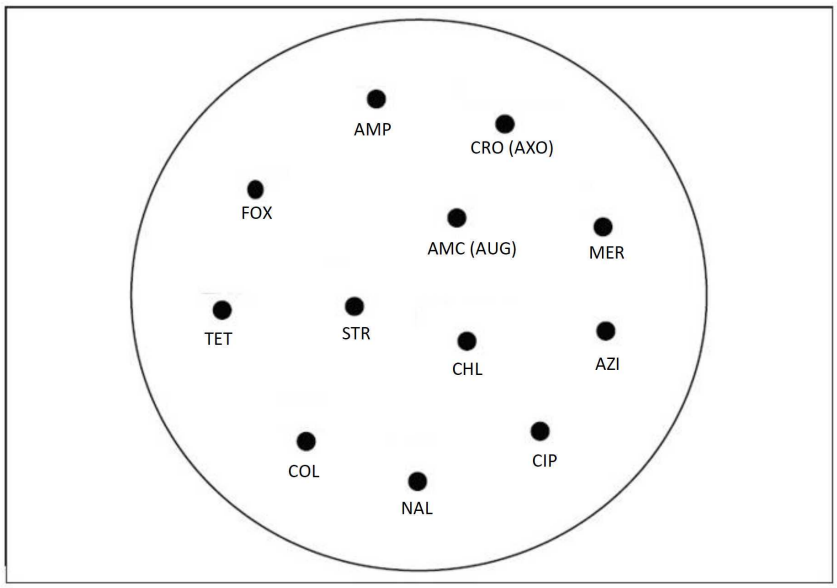
51 Remove all blood agar-plates from the freezer

52 Follow the Clinical and Laboratory Standards Institute (CLSI)'s guidelines for antibiotic susceptibility testing using the disk diffusion method

CLSI (2018). Performance Standards for Antimicrobial Susceptibility Testing. 28th ed. CLSI supplement M100. Clinical and Laboratory Standards Institute.

Apply antibiotic disks on Muller Hinton agar according to the plate1 map bellow

PLATE 1



AMC: amoxicillin-clavulanic acid 20/10µg
AMP: ampicillin 10µg
AZI: azithromycin 15µg
FOX: cefoxitin 30µg
CRO: ceftriaxone 30µg
CHL: chloramphenicol 30µg
CIP: ciprofloxacin 5µg
COL: colistin 10µg
MER: meropenem 10µg
NAL: nalidixic acid 30µg
STR: streptomycin 10µg
TET: tetracycline 30µg

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

DAY 3 : Antibiotic susceptibility testing

53 - After the antimicrobial susceptibility testing

- Record diameters of inhibition zones in mm

54 Compare inhibition zone diameters to the CLSI clinical breakpoints to classify bacterial isolates as resistant, intermediate or susceptible.

For Colistin : Use breakpoint by Galani et al., 2008

Galani I, Kontopidou F, Souli M, Rekatsina P, Koratzanis E, Deliolanis J, et al. (2008). Colistin susceptibility testing by Etest and disk diffusion methods.. Int J Antimicrob Agents. http://doi:10.1016/j.ijantimicag.2008.01.011

55 **For *E.coli***

1. Antibiotic resistance results for non-type-specific *E.coli* Isolated from MacConkey agar without antibiotics: ***E.coli* isolate-level prevalence of resistance to 12 antibiotics.**

2. Antibiotic resistance results for presumptive *E.coli* resistant to third-generation cephalosporins isolated from MacConkey agar with cefotaxime: **Confirmed *E.coli* resistant to third-generation cephalosporins**

- Proportion of samples with *E.coli* resistant to third-generation cephalosporins

- Calculate the **sample-level prevalence for third-generation cephalosporin resistance** :

$$\frac{\text{Number of samples with Confirmed } E.coli \text{ resistant to third-generation cephalosporins}}{\text{Total number of samples collected}}$$

- Establish antibiotic resistance profiles of third-generation cephalosporin resistant *E.coli*

3. Antibiotic resistance results for presumptive *E.coli* resistant to quinolones isolated from MacConkey agar with ciprofloxacin: **Confirmed *E.coli* not susceptible (resistant and intermediate) to quinolones**

- Proportion of samples with *E.coli* not susceptible to quinolones

- Calculate the **sample-level prevalence for low susceptibility to quinolone** :

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

Number of samples with Confirmed *E.coli* not susceptible to quinolones

---

Total number of samples collected

- Establish antibiotic resistance profiles of *E.coli* with low susceptibility to quinolones

For *Salmonella*

1. Antibiotic resistance results for Non-type-specific *Salmonella* isolated from agar media without antibiotics: ***Salmonella* isolate-level prevalence of resistance to 12 antibiotics.**

2. Antibiotic resistance results for presumptive *Salmonella* resistant to third-generation cephalosporins isolated from agar media with cefotaxime: **Confirmed *Salmonella* resistant to third-generation cephalosporins**

- Proportion of samples with *Salmonella* resistant to third-generation cephalosporins

- Calculate the **sample-level prevalence for third-generation cephalosporin resistance** :

Number of samples with Confirmed *Salmonella* resistant to third-generation cephalosporins

---

Total number of samples collected

- Establish antibiotic resistance profiles of third-generation cephalosporin resistant *Salmonella*

3. Antibiotic resistance results for presumptive *Salmonella* not susceptible to quinolones(resistant and intermediate) isolated from agar media with ciprofloxacin: **Confirmed *Salmonella not susceptible* to quinolones**

- Proportion of samples with *Salmonella* not susceptible to quinolones

- Calculate the **sample-level prevalence for low susceptibility to quinolone** :

Number of samples with Confirmed *Salmonella* not susceptible to quinolones

---

Total number of samples collected

- Establish antibiotic resistance profiles of *Salmonella* not susceptible to quinolones

56 Identify all *E.coli* and *Salmonella* isolates resistant to third-generation cephalosporins despites the media of isolation

57 These isolates will be tested for beta-lactamases production using the combination disk test according to CLSI guidelines

58 Streak identified third-generation cephalosporin resistant *E.coli* and *Salmonella* isolates on 5% sheep blood agar plates

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

59 Incubate all inoculated plates at **37 °C** **Overnight**

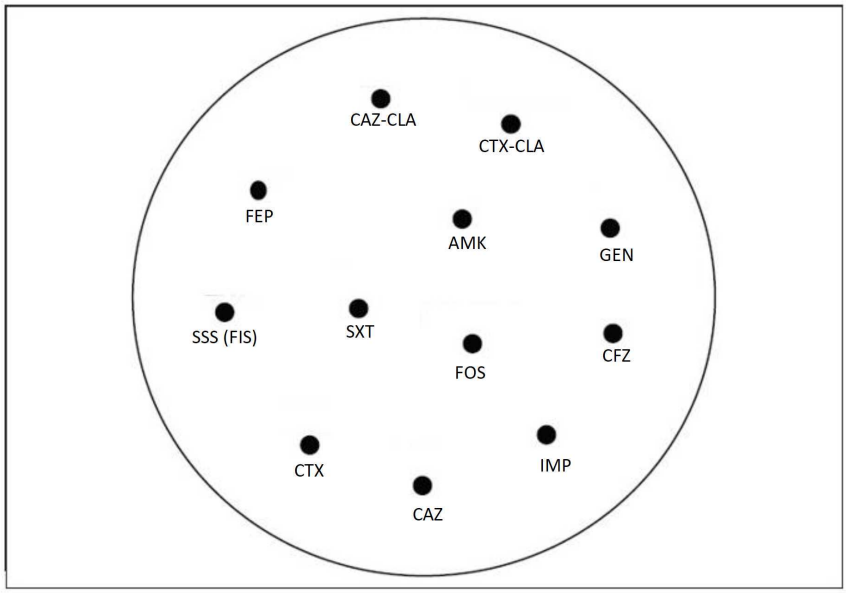
DAY 4 : Antibiotic susceptibility testing

60 Follow the CLSI protocol for the combination disk test to detect the production of extended spectrum beta-lactamases (ESBLs)

CLSI (2018). Performance Standards for Antimicrobial Susceptibility Testing. 28th ed. CLSI supplement M100. Clinical and Laboratory Standards Institute.

Apply antibiotic disks on Muller Hinton agar according to the plate 2 map

PLATE 2



CTX: cefotaxime 30µg
CTX-CLA: cefotaxime-clavulanic acid 30/10µg
CAZ: ceftazidime 30µg
CAZ-CLA: ceftazidime-clavulanic acid 30/10µg
AMK: amikacin 30µg
CFZ: cefazolin 30µg
FEP: cefepime 30µg
FOS: fosfomycin 200µg
GEN: gentamicin 10µg
IMP: imipenem 10µg
SSS: sulfisoxazole 300µg
SXT: trimethoprim/sulfamethoxazole 1.25/23.75µg

DAY 5 : Antibiotic susceptibility testing

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

61 - After the combination disk test

- Record diameters of inhibition zones in mm

62 Compare inhibition zone diameters to the CLSI clinical breakpoints to classify bacterial isolates as resistant, intermediate or susceptible.

63 Identify ESBL or AmpC producing bacterial isolates

- ESBL producers: If the presence of clavulanic acid increases the inhibition zone diameters by at least 5 mm for either ceftazidime or cefotaxime, then the test is considered positive for the production of an ESBL.
- AmpC producers: If the presence of clavulanic acid doesn't increase the inhibition zone diameters by at least 5 mm for either ceftazidime or cefotaxime, then the test is considered positive for the production of an AmpC.

Precaution while deciding if a bacterial isolate has an ESBLs or AmpC phenotype:
The combination disk test can produce several false positive *E. coli* producing extended spectrum beta-lactamases, especially when the results (difference in inhibition zone diameter caused by the clavulanic acid) are close to 5 mm decision point.

Poulou A, Grivakou E, Vrioni G, Koumaki V, Pittaras T, Pournaras S, Tsakris A (2014). Modified CLSI extended-spectrum β-lactamase (ESBL) confirmatory test for phenotypic detection of ESBLs among Enterobacteriaceae producing various β-lactamases.. Journal of clinical microbiology.
https://doi.org/10.1128/JCM.03361-13

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw

Robberts FJ, Kohner PC, Patel R (2009). Unreliable extended-spectrum beta-lactamase detection in the presence of plasmid-mediated AmpC in Escherichia coli clinical isolates.. Journal of clinical microbiology.
https://doi.org/10.1128/JCM.01687-08

Citation: Rosine Manishimwe, Paola M. Moncada, Marie Bugarel, H. Morgan Scott, Guy H. Loneragan (05/08/2021). Isolation and phenotypic characterization of antibiotic resistant E.coli and Salmonella from food animal feces. https://dx.doi.org/10.17504/protocols.io.brjkm4kw