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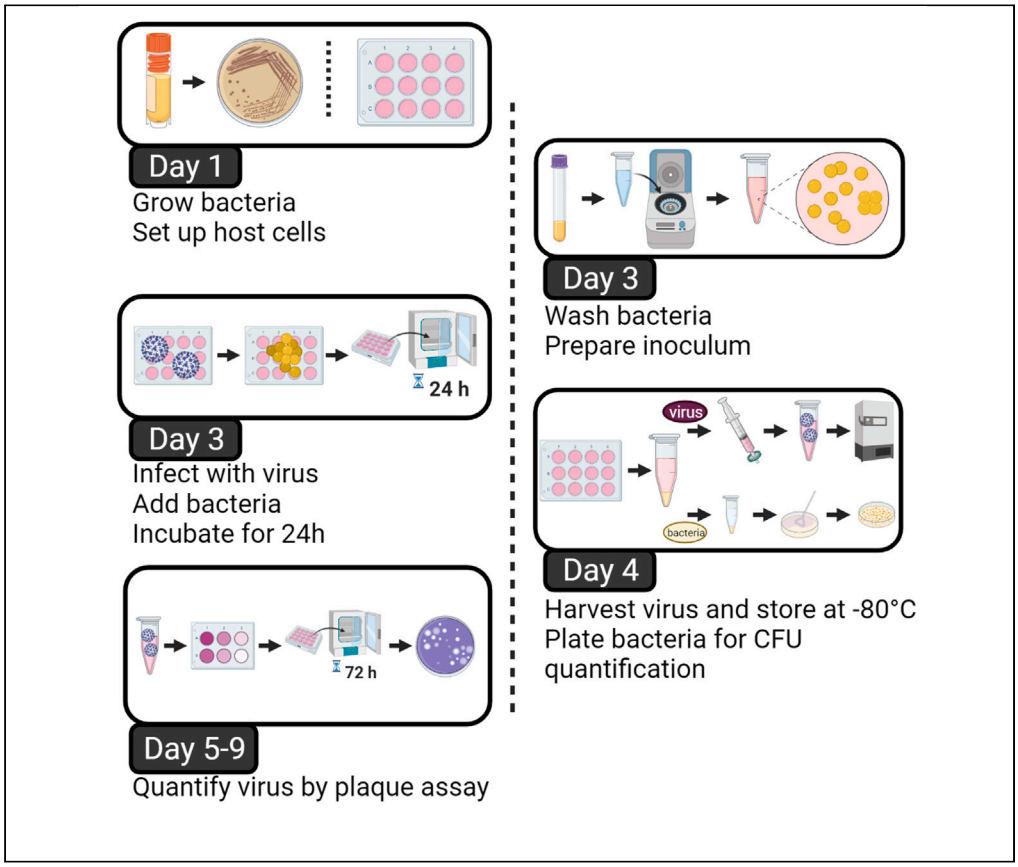
Protocol for studying co-infection between SARS-CoV-2 and *Staphylococcus aureus* *in vitro*



Bacterial co-infection is one of the most common complications of SARS CoV-2 infection. Here, we present a protocol for the *in vitro* study of co-infection between SARS CoV-2 and *Staphylococcus aureus*. We describe steps for quantifying viral and bacterial replication kinetics in the same sample, with the optional extraction of host RNA and proteins. This protocol is applicable to many viral and bacterial strains and can be performed in different cell types.

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

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Highlights

In vitro technique to study co-infection between SARS CoV-2 and *Staphylococcus aureus*

Simultaneous quantification of viral and bacterial replication

Steps to perform parallel measurements of host RNA and/or protein

Compatible with different viral and bacterial strains

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Protocol for studying co-infection between SARS-CoV-2 and *Staphylococcus aureus in vitro*

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SUMMARY

Bacterial co-infection is one of the most common complications of SARS CoV-2 infection. Here, we present a protocol for the *in vitro* study of co-infection between SARS CoV-2 and *Staphylococcus aureus*. We describe steps for quantifying viral and bacterial replication kinetics in the same sample, with the optional extraction of host RNA and proteins. This protocol is applicable to many viral and bacterial strains and can be performed in different cell types.

For complete details on the use and execution of this protocol, please refer to Goncheva et al.[1]

BEFORE YOU BEGIN

One of the most common complications of primary SARS-CoV-2 infection is secondary bacterial co-infection. Data from across the world has shown that co-infection most frequently occurs with the Gram-positive bacterium *Staphylococcus aureus*.[2–7] As the clinical presentation is viral infection first, followed by bacterial co-infection, we developed an assay that mimics these events. The protocol below describes an *in vitro* co-infection assay that allows monitoring of the replication kinetics of both virus and bacteria, as well as measurement of host RNA or proteins. The protocol uses the original strain of SARS-CoV-2 but can also be used with any other new variants. We describe the protocol for Vero E6 cells, but other virus permissive cell lines can also be used, including Vero cells or primary human lung epithelial cells. Although the protocol was developed with the *S. aureus* strain USA300, any strain and/or mutant can be used instead.

Biosafety

This protocol combines two different containment levels.

1. Work with SARS-CoV-2 currently requires the use of a containment level 3 (CL3) laboratory. Training and usage will be specific to the research institution. Follow all local and national guidelines for work in high-containment laboratories.
2. Work with *S. aureus* requires the use of a containment level 2 (CL2) laboratory. Training and usage will be specific to the research institution.

Institutional permissions

Institutional permission for operation of and within a Containment Level 3 laboratory and associated national level certifications.

Mammalian cell maintenance

Timing: 7 days

Vero E6 cells

Note: Instructions for the maintenance of cells are available from the ATCC supplier: https://www.atcc.org/products/crl-1586. We have provided a brief version here for ease.

3. Prepare the cell culture complete media. Add 55.5 mL of Fetal Bovine Serum (FBS) to a bottle (500 mL) of Dulbecco's Modified Eagle's Medium (DMEM). Store at 4°C for up to a month.
4. Thaw Vero E6 cells.
   a. Pre-warm all reagents in a 37°C water bath for 1 h.
      i. Add 45 mL of complete media to a 50 mL centrifuge tube.
      ii. Place 5 mL into a 25 cm^2 tissue culture flask and the remaining 40 mL in a 50 mL centrifuge tube.
   b. Retrieve an aliquot of frozen cells from liquid nitrogen.
   c. Place the aliquot in the 37°C water bath until it has thawed (approx. 1 min).
      i. Move the tube into a Biological Safety Cabinet (BSC).
      ii. Using sterile technique carefully transferred the cells on the 40 mL of complete media from step a).
      iii. Centrifuge the tube at 250 × *g* for 3 min.
   d. In the Biological Safety Cabinet (BSC), discard the supernatant, taking care not to disturb the pellet.
      i. Take 1 mL of complete media from the 25 cm^2 tissue culture flask and use it to gently re-suspend the pelleted cells.
      ii. Return the cell containing media into the tissue culture flask.
      iii. Place the flask in an incubator at 37°C, 5% CO_2 and incubate for 1–2 days, until confluent.
5. Maintain Vero E6 cells. Once cells have reached 90%–100% confluence, they need to be passaged.
   a. Pre-warm 1× PBS, complete media and 0.25% Trypsin/EDTA in a 37°C water bath for 1 h.
   b. In a BSC, remove the media from the flask and wash twice with 1× PBS.
   c. Add 2 mL of 0.25% Trypsin/EDTA and return the flask to the 37°C, 5% CO_2 incubator for 2–3 min. Gently tap the side of the flask to release the cells.
   d. Add an equal volume (2 mL) of complete media to neutralize the trypsin.
      i. Transfer the solution to a centrifuge tube.
      ii. Centrifuge at 250 × *g* for 3 min.
   e. Re-suspend the cells in 5 mL of complete media.
      i. Use 1/10^{th} (500 µL) to seed a new flask. If desired, you can set up larger flasks – adjust the seeding density depending on the size.
      ii. Place the flask in an incubator at 37°C, 5% CO_2 and incubate for 4–5 days, until confluent.

⚠ CRITICAL: Cells need to be maintained in antibiotic free media to allow for the subsequent growth of bacteria during infection.

Note: Cells were not used past passage number 40.

Virus

SARS-CoV-2 USA-WA1/2020 strain was obtained from BEI resources. Virus was amplified on Vero E6 cells (designated as passage 2), aliquoted and stored at −80°C. Passage 2 virus was amplified again on Vero E6 cells (designated as passage 3), aliquoted and stored at −80°C. Passage 2 or passage 3 virus stocks were used for all experiments.

Bacteria

Staphylococcus aureus strain USA300 was obtained from BEI resources. Bacteria were streaked on a Tryptic Soy Agar (TSA) and incubated at 37°C for 16–18 h. Bacteria were scraped from the plate using a metal loop and re-suspended in 700 µL of TSB in a 2 mL cryovial. 700 µL of sterile 30 w/v were added and the mixture vortexed extensively. Bacteria were stored at −80°C. Bacteria were freshly

streaked on a TSA plate for each experiment (see step 7 below). *S. aureus* mutants were generated as described previously.[1]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
SARS-CoV-2 USA-WA1/2020	BEI	https://www.beiresources.org/Catalog/animalviruses/NR-52281.aspx
Staphylococcus aureus	BEI	https://www.beiresources.org/Catalog/Bacteria/NR-46543.aspx
Chemicals, peptides, and recombinant proteins		
2× MEM	Gibco	11935046
Fetal bovine serum	Wisent	080-150
Pen/strep stock	Wisent	450-201-EL
HEPES (1 M)	Fisher	15630-080
L-Glutamine	Fisher	25030081
Methanol	Caledon	6701-2-40
Crystal violet powder	Sigma	C0775
Cellulose powder	Sigma	435244
Tryptic soy broth	Wisent	800-056-CG
Agar	Wisent	800-010-CG
Dulbecco's modified Eagle's medium (DMEM)	Wisent	319-007-CL
0.25% Trypsin/EDTA	Wisent	325-043-EL
10% Neutral buffered Formalin	Fisher	23245684
RNA protect cell reagent	Qiagen	76526
RIPA buffer	Fisher	PI89900
cOmplete mini protease inhibitor, EDTA-free	Sigma	11836170001
Critical commercial assays		
RNeasy Mini Kit	Qiagen	74106
Experimental models: Cell lines		
Vero E6 cells	ATCC	https://www.atcc.org/products/crl-1586
Other		
Sterile 1 mL syringe	Fisher	B309659
13 mm 0.22 µm Nylon sterile syringe filters	Mandel	229760
Tissue culture plastics: - 12 well plates - 6 well plates - 25 cm^2 flasks - 175 cm^2 flasks	Various suppliers	

MATERIALS AND EQUIPMENT

2× plaque assay media

Store at 4°C for up to 4 weeks.

Reagent	Final concentration	Amount
2× MEM (Gibco 11935046)	N/A	455 mL
Fetal bovine serum (Wisent 080-150)	4%	20 mL
Pen/strep stock (Wisent 450-201-EL)	2%	10 mL
HEPES (1 M) (Fisher 15630-080)	2%	10 mL
L-Glutamine (Fisher 25030081)	1%	5 mL
Total	N/A	500 mL

Alternatives: Other suppliers of FBS, Pen/Strep, HEPES and L-Glutamine can be used, so long as they are suitable for tissue culture and are sterile.

Note: We routinely use FBS that has NOT been heat inactivated. While we have not done a side-by-side comparison between FBS that has and has not been heat inactivated, we do not foresee any problems in performing this protocol with heat inactivated FBS.

Crystal Violet stain

Store at 20°C–23°C indefinitely.

Reagent	Final concentration	Amount
H_2O	N/A	400 mL
Methanol	20% (v/v)	100 mL
Crystal Violet powder (Sigma C0775)	1% (w/v)	5 g
Total	**N/A**	**500 mL**

Alternatives: Other suppliers of Crystal Violet powder can be used.

⚠ CRITICAL: Methanol is hazardous. Handle concentrated solution only in a fume hood.

Cellulose overlay

- 488 mL H_2O.
- 12 g cellulose powder (Sigma 435244).

Stir vigorously for 2–3 h. Autoclave and store at 20°C–23°C for up 6 months.

Tryptic Soy Broth

- 480 mL H_2O.
- 15 g Tryptic Soy Broth (TSB) powder (Wisent 800-056-CG).

Stir until the powder dissolves. Aliquot 5 mL into 30 mL glass tubes. Place lid. Autoclave. Store at 20°C–23°C for up to 4 weeks.

Alternatives: You can use another supplier for TSB.

Tryptic Soy Agar plates

Reagent	Final concentration	Amount
H_2O	N/A	480 mL
TSB	N/A	15 g
Agar	1.5% (w/v)	55 g
Total	**N/A**	**500 mL**

Place in an Erlenmeyer flask of at least double the volume. Autoclave and pour into petri dishes while still liquid. Store set agar plates at 20°C–23°C for up to 4 weeks.

Alternatives: You can use another supplier for TSB and agar powder. Or you can use ready-made Tryptic Soy Agar powder.

Vero E6 culture media

Add 55.5 mL of FBS to a bottle of DMEM under sterile conditions. Store at 4°C for up to a month.

- 500 mL Dulbecco's Modified Eagle's Medium (DMEM) (Wisent 319-007-CL).
- 55.5 mL Fetal bovine serum (FBS).

Alternatives: Other suppliers of DMEM can be used. Use your choice of supplier for FBS.

Other required consumables

Reagent	Catalog number
Sterile 1 mL syringe	Fisher B309659
13 mm 0.22 µm Nylon sterile syringe filters	Mandel 229760
10% Neutral buffered Formalin	Fisher 23245684
Tissue culture plastics: - 12 well plates - 6 well plates - 25 cm^2 flasks - 150 cm^2 flasks	Any plastics for use with tissue culture.

Alternatives: Other sources of 1 mL syringes and neutral buffered formalin can be used. The choice of manufacturer for tissue culture plastics is up to the user.

STEP-BY-STEP METHOD DETAILS

Grow the SARS-CoV-2 virus

Timing: 9 days

This step will provide you with virus stocks for use in co-infection assays. Previously generated stocks can also be used, provided they have been grown without antibiotics.

Note: All work in steps 4 and 5 should be performed in a CL3 laboratory.

1. Day 1 – Set up cells as per steps 1–3.
   a. Prepare 175 cm^2 flasks of Vero E6 cells, seeding them so that they would be 95%–100% confluent in 48 h. Include an additional 25 cm^2 flask to be used as a negative control (mock infection). Seed flasks with at 1 × 10^5 cells/mL, using 5 mL for the 25 cm^2 flask and 35 mL for the 175 cm^2 flask.

   Note: Vero E6 cells are used for most variants of SARS-CoV-2. However, if you're using Omicron variants, you should use Vero cells instead.

2. Day 3 – Infect cells.
   a. Pre-warm all reagents at 37°C in a water bath or incubator.
   b. Dilute your virus stock to a multiplicity of infection of 0.01 in 5 mL of DMEM (without any supplements). A confluent T175 cm^2 flask will have approx. 3 × 10^7 cells. Use this number and the equation below to calculate the amount of virus to be used:
      (MOI) * (number of cells) / virus stock concentration in PFU/mL.
   c. Remove media from flasks.
      i. Wash cells twice with 1× PBS. Add the 5 mL of virus calculated as per step 5.b.
      ii. Set up a "mock" infection in the 25 cm^2 flask, where you add 2 mL of DMEM but NO virus stock.
      iii. Return the flasks to 37°C, 5% CO_2 for 1 h.

iv. Every 10 min gently rock flasks to re-distribute the inoculum and prevent cells from drying out.

d. After 1 h, remove the viral inoculum and wash cells once with 1× PBS.
   i. Add 35 mL of DMEM + 2% FBS.
   ii. Incubate at 37°C, 5% CO_2 for 72 h, or until ~90% cytopathic effect (CPE) is visible.

Note: CPE for this virus can be described as fusion of neighboring cells into syncytia and cell death leading to the appearance of gaps in the cell monolayer. No CPE should be seen in your mock flask.

e. Harvest the media from the flasks.
   i. Centrifuge at 500 × *g* for 10 min at 4°C.
   ii. Transfer the resulting supernatant into a new tube.
   iii. Aliquot and store at −80°C.

Pause point: Once viral aliquots have been placed in −80°C, they can be stored until you have the time to perform plaque assays.

3. Day 4–9 Measure virus titer by plaque assay.
   a. Day 4 - two days before the plaque assay, set up 6 well plates of Vero E6 cells as per steps 1–3. Use a cell density that will ensure that plates are 100% confluent 48 h later. Seed cells at 5 × 10^4 cells/mL, using 2 mL of media per week for 6 well plates.
   b. Day 6 – Set up plaque assay.
      i. Set up 10-fold serial dilutions of your virus samples from step 4.d in DMEM (without any supplements).
      ii. In 1.5 mL microcentrifuge tubes, add 450 μL of DMEM and 50 μL of virus stock. Pipette up and down to mix. Discard the tip. Using a sterile tip, take 50 μL from the tube and place it into a new tube containing 450 μL of DMEM. Continue until a 10^{-7} dilution.
      iii. Take 6 well plates out of the incubator. Wash once with 1× PBS. Add 400 μL of virus dilution per well. Return the plate to 37°C, 5% CO_2 for 1 h. Every 10 min gently rock plates to re-distribute the inoculum and prevent cells from drying out.
      iv. While the plates are incubating, prepare your overlay. Mix a ratio of 1:1 of DMEM and 2.4% cellulose in a 50 mL centrifuge tube. Invert vigorously until a uniform suspension has formed. Place the tubes in a 37°C water bath to pre-warm. You will need 12 mL of overlay for each 6 well plate.
      v. Remove plates from the incubator and place them in the BSC. Take your overlay tubes from the water bath and place them in the BSC. Remove the inoculum from the 6 well plates. Invert the overlay tubes to ensure the suspension is until uniform. Pipette 2 mL per well of the overlay using a serological pipette. Incubate plates at 37°C, 5% CO_2 for 3 days. Ensure plates are not moved during this time.

Note: We do not perform wash steps after the inoculum is removed (step 6.a.iv). We based our protocol on similar protocols described for plaque assays of SARS-CoV-2[8] and Influenza,[9] which also do not include a wash step before addition of the overlay.

   c. Day 9 – Fix and stain the plaque assay.

Note: All the steps should be performed in a BSC, until you are ready to count plaques (step 6.c.v)

      i. Remove the plates from the incubator and place them in the BSC. Add 2 mL per well of 10% Neutral Buffered formalin (NBF) using a serological pipette. Gently swirl the plates until you can see the cellulose mixture lift up and the NBF is in full contact with the cells. Incubate at 20°C–23°C for 30 min.

ii. Remove the NBF mixture into a waste basin. Rinse the cells once-twice with water.
iii. Add 1 mL of Crystal Violet stain (see materials) per well. Incubate at 20°C–23°C for 20 min.
iv. Remove the Crystal Violet stain. Rinse the cells twice with water. The volume of water does not need to be measured and is most easily done with a wash bottle. Tap excess water on a paper towel. Allow plates to dry in the hood for 15–30 min.
v. Remove plates and count plaques.
Calculate plaque forming units per mL using the following formula: PFU/mL = (number of plaques x 2.5) x (dilution factor).

Note: The volume of water does not need to be measured and is most easily done with a wash bottle.

⚠ CRITICAL: Stocks MUST be grown without the presence of antibiotics, in order to prevent residual antibiotics from preventing *S. aureus* growth.

Grow *S. aureus*

Timing: 3 days

This step provides you with bacterial inoculum that can be used in co-infection assays. The inoculum must be freshly prepared on the day of the infection. The time for that has been included in the co-infection protocol (see below).

4. Prepare the bacterial inoculum.
   a. Day 1 - Prepare single colony streak plates.
      i. Streak *S. aureus* bacteria from frozen glycerol stocks on 100 mm × 15 mm Tryptic Soy Agar (TSA) plates. If you are using mutants or strains containing plasmids, include appropriate antibiotics in the agar. Incubate plates at 37°C for 16–18 h.
   b. Day 2 - Prepare 16–18 h cultures.
      i. Take a single colony of bacteria and inoculate into 5 mL of Tryptic Soy Broth (TSB) in a 30 mL tube. If you are using mutants or strains containing plasmids, include appropriate antibiotics in the broth. Incubate at 37°C, 200 rpm for 16–18 h.
   c. Day 3 – Prepare bacterial inoculum for cell infection.
      i. Transfer 500 μL of bacteria into a 1.5 mL microfuge tube. Spin at 20,000 × *g* for 1 min.
      ii. Wash twice with 700 μL of DMEM. Spin at 20,000 × *g* for 1 min between washes.
      iii. Re-suspend bacteria in 500 μL of DMEM.
      iv. Measure Optical Density (OD) at 600 nm.
      v. In a separate tube, dilute your bacteria to a final OD_{600} of 0.33 in 500 μL of DMEM. Take a sample of this solution to confirm the correct number of bacteria are present.
      vi. To determine bacterial numbers, use a 96 well plate.
      vii. Take 20 μL of the solution you want to quantify and place it in 180 μL of 1× PBS in well 1. Pipette up and down to mix. Discard the tip. Using a sterile tip, take 20 μL from well 1 and place it into a new well containing 180 μL of 1× PBS. Continue until a 10^{-7} dilution. Take 10 μL of each dilution and spot them on a 100 mm × 15 mm TSA plate. Repeat the plating step twice, so that you have two replicates plated for each dilution. Incubate plates at 37°C for 16–18 h. After incubation, select the dilution where 10–40 colonies are present and count the number of colonies. Take the average count from the two repeats you plated. Use this average value to calculate CFU/mL using the following formula:
      CFU/mL = (number of colonies) x (dilution factor)/volume plated (mL).
      viii. Use 5 μL of the solution (OD_{600} of 0.33) from step 7.c.v in a well of a 12 well tissue culture plate. This should be the equivalent of 1 × 10^5 CFU/well.

Viral bacterial co-infection and pathogen quantification

Timing: 10 days

This step comprises the viral – bacterial co-infection assay. The outline of the experiments is summarized in Figure 1. The assay relies on previously prepared cells, virus and bacteria. It allows for the quantification of viral and bacterial replication, and assessment of host RNA and/or protein, depending on your experimental needs. Different time points post infection can be used if desired.

5. Day 1 - Plate host cells in 12 well plates as per steps 1–3, and streak bacteria strains.
   a. For 12 well plates, cells are routinely seeded at 5×10^4 cells/mL, which results in confluent wells in 48 h. Use 1 mL of media per well for 12 well plates.
   b. Streak bacteria strains as per step 7a.
6. Day 2 - Prepare 16–18 h bacterial cultures as per step 7.b.
7. Day 3 – Infect cells with SARS-CoV-2 and *S. aureus.*

   Note: All work from this step onwards should be performed in a CL3 laboratory.

   a. Prepare your bacterial inoculum as per step 7c. From this step forward, work should continue in a CL3 laboratory.
   b. Prepare viral inoculum. For a confluent 12 well plate, assume a cell population of 1×10^5 cells per well. Dilute virus stocks for a multiplicity of infection (MOI) of 1 in a volume of 100 µL per well of DMEM (see step 5.b for the formula for MOI calculations).
   c. Remove media from 12 well plates. Wash them twice with 1× PBS at approx. 1 mL per well. Remove PBS and add 100 µL of the virus inoculum prepared in step 10.b per well. Ensure you have a "mock" well, where only 100 µL of DMEM, but no virus, are added. Return the plate to 37°C, 5% CO_2 for 1 h. Every 10 min gently rock plates to re-distribute the inoculum and prevent cells from drying out.
   d. Remove plates from the incubator. Take the viral inoculum off. Wash wells twice with 1× PBS. Add 1 mL of DMEM to each well.
   e. To desired wells, add 5 µL of bacterial inoculum, as prepared in step 7.c. For each infection, you should have control samples of mock, including virus alone and bacteria alone. If you are testing more than one bacterial strain and/or mutant, ensure you have bacteria only wells for each strain, in order to facilitate bacterial counts.
   f. Incubate plates at 37°C, 5% CO_2 for 24 h.
8. Day 4 – Harvest infections for bacteria and virus quantification.
   a. Retrieve the plates from the incubator. Harvest the culture media into 1.5 mL microfuge tubes. Spin at 15,000 × *g* for 1 min.
   b. While samples are spinning, add 500 µL of 1× PBS + 0.1% (v/v) Triton X-100 to all wells.
   c. Once centrifugation is complete, take the microfuge tubes back into the hood. Using a 1 mL syringe, gently take the supernatant from the microfuge tube, without disturbing the bacterial pellet. Attach the syringe to a 13 mm, 0.22 µm filter. Filter the supernatant into a new 1.5 mL microfuge tube. These supernatants can now be used for the quantification of virus. They can be measured immediately or stored at −80°C until needed.
   d. Harvest the cell lysates in the 1× PBS + 0.1% (v/v) Triton X-100 from the wells in step 11.b and add it to the remaining pellets from step 11.c. Pipette up and down a few times to re-suspend the pellet. Perform a serial dilution of this sample (cell lysate and bacterial pellet) and plate on TSA plates for CFU enumeration, as described in step 7.c.v. Incubate plates at 37°C for 18 h.

Pause point: Once viral samples have been placed in −80°C, they can be stored until you have the time to perform plaque assays or other measurement techniques.

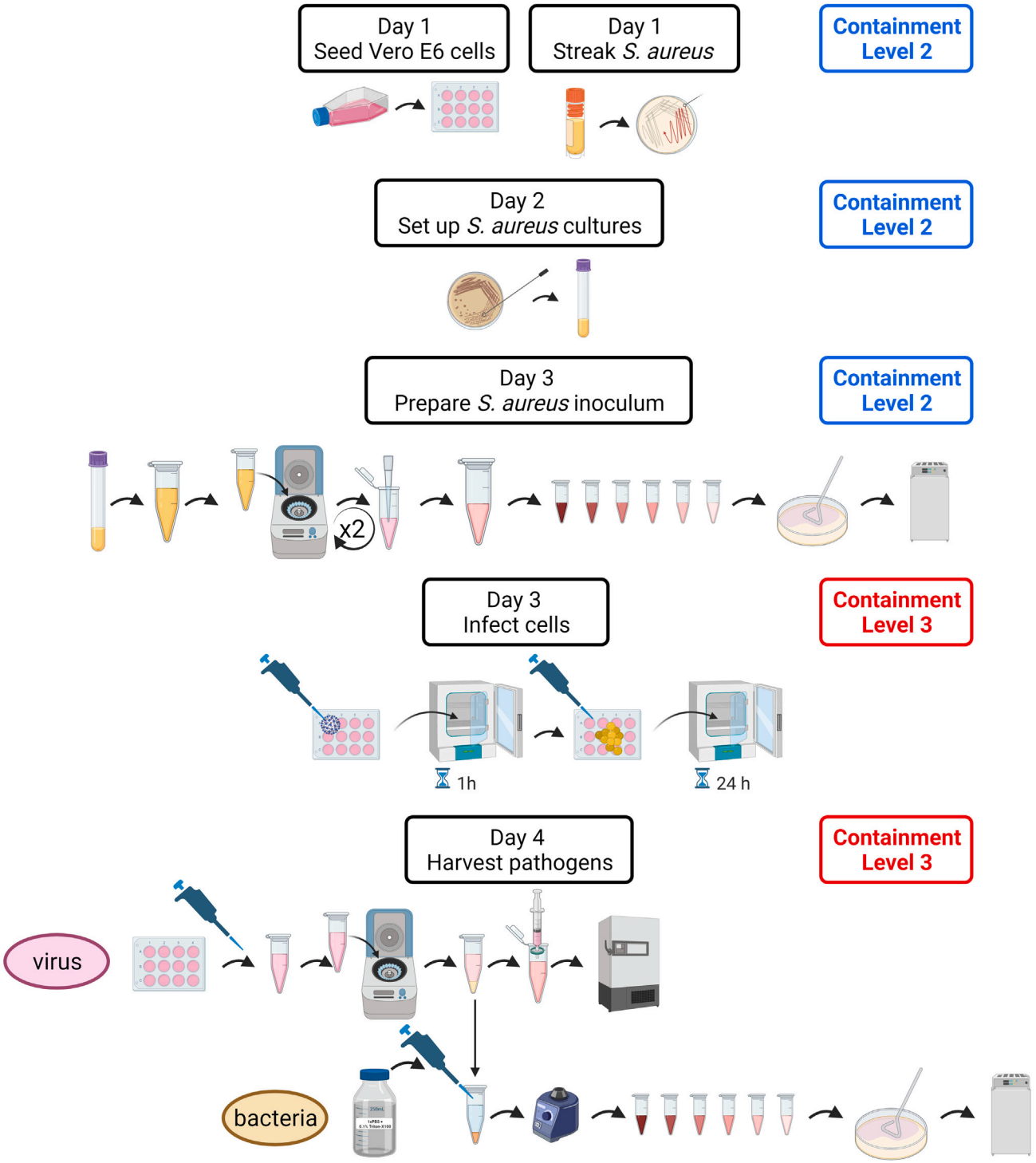

Figure 1. Schematic of the steps during the 4 days of a SARS-CoV-2 – *S. aureus* co-infection assay

The figure demonstrates the main components of the assay – when and how to set up host cells, bacterial cultures and virus infection. Image created using Biorender.

Note: If you encounter difficulties with bacterial growth, refer to common problems and solutions in the troubleshooting section.

Optional: Day 4 – Harvest cellular RNA or protein. The protocol allows users to perform host RNA or protein isolation if desired. For this, steps 8–10 are performed as above. Following the incubation described in step 10f, retrieve the plates and place them in a BSC. Follow the steps below for RNA or protein isolation. All work in this step should be performed in a CL3 laboratory and in a BSC.

Note: If users wish, they can still perform a plaque assay and quantify the amount of virus from samples that will later be used for RNA or protein purification. If you wish to determine PFU/mL in parallel to RNA/protein, perform steps 11a and 11c with the supernatant, and then proceed to use the cells as described below. Please note it is NOT possible to measure bacterial CFU for RNA/protein samples.

e. Host RNA isolation.
   i. Infect cells as outlined in steps 8–1o. Following the desired incubation time, remove culture supernatant and wash cells three times with 1× PBS. Add 500 µL of Cell Protect reagent (Qiagen) to each well and incubate at RT for 5 min. Harvest the wells and centrifuge at 1,200 × *g* for 5 min. Remove the supernatant and store the pellets at −80°C.
   ii. Lyse cell pellets with 500 µL of 1× PBS + 0.1% (v/v) Triton - X100 (see Note below). Proceed with the RNA extraction using the QIAGEN RNEasy kit, as per the manufacturer's instructions. Manufacturer's instructions can be found here: https://www.qiagen.com/ca/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en . For ease of use, we have also included a brief summary of the RNA extraction protocol here (spin version).
   iii. Add 600 µL of buffer RLT to the re-suspended cell pellets from the previous step. Pipette to mix.
   iv. Add 1 volume (1.1 mL) of 70% ethanol to the cell lysate and mix well by pipetting.
   v. Transfer 700 µL of the sample from step iv to an RNeasy spin column and centrifuge for 1 min at 10 000 x *g*. Discard the flow through.
   vi. Repeat step v until you have passed all the lysate through the column.
   vii. Add 700 µL of buffer RW1 to the column. Centrifuge for 1 min at 10 000 × *g*. Discard the flow through.
   viii. Add 500 µL of buffer RPE to the column. Centrifuge for 2 min at 10 000 × *g*. Discard the flow through.
   ix. Centrifuge for 2 min at 10 000 × *g*. Discard the flow through.
   x. Place the RNeasy column into a new 1.5 mL collection tube. Add 40 µL of water to the membrane. Incubate for 1 min at RT. Centrifuge for 1 min at 10 000 × *g*.
   xi. Repeat step x. for a second elution (use the same tube).
   xii. You may now remove these samples from CL3 and process the protein as desired. Please follow appropriate decontamination protocols for removing samples from a CL3 laboratory, as per your local SOPs.
   xiii. Store RNA samples at −80°C

Note: We had previously optimized this step for the isolation of RNA from cells infected with bacteria. We determined that the use of 500 µL of PBS + 0.1% v/v Triton X-100 (step a.ii above) improved yield if performed before the addition of RLT buffer and have since included it in all of our RNA isolation protocols. If users desire, they can skip this re-suspension step and proceed directly to the addition of the RLT buffer (step a.iii above).

f. Host protein isolation.**

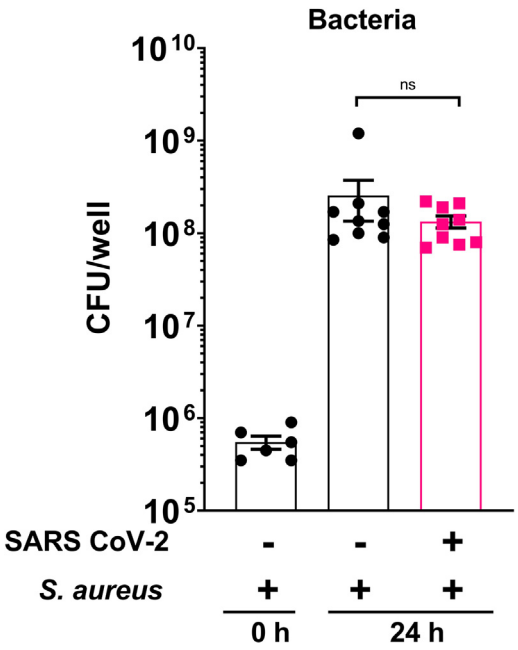

Figure 2. *S. aureus* replication occurs over the 24 h of co-infection

Vero E6 cells were mock infected or infected with CoV-2 at MOI of 1 for 1 h and overlayed with 1 mL of serum free DMEM. 1 × 10^5 CFU of WT *S. aureus* strain USA300 were added to the culture media. 24 h later, DMEM was harvested, samples were centrifuged for 1 min at 15000 × *g*. 500 μL PBS + 0.1% v/v Triton X-100 were added to the tissue culture wells. Following centrifugation, supernatant was removed, and the pellet resuspended in the 500 μL PBS + 0.1% Triton X-100 from the wells. Bacteria were serially diluted and plated on TSA plates. Plates were incubated at 37°C for 18 h and CFU enumerated. Data shown are mean ± SEM of 3-5 independent experiments. Figure re-printed with permission from Goncheva et al.[1]

i. Infect cells as outlined in steps 8–10 . Following the desired incubation time, remove culture supernatant and wash cells three times with 1× PBS. Add 50 μL of RIPA buffer with protease inhibitor to each well. Incubate samples on a rocker at 4°C for 30 min.

ii. Centrifuge samples at 12,000 × *g* for 20 min. Harvest the resulting supernatant and place it in a new tube. You may now remove these samples from CL3 and process the protein, as desired. Please follow appropriate decontamination protocols for removing samples from a CL3 laboratory, as per your local SOPs.

9. Day 5 – Count bacteria.
   a. Remove plates from step 11.e from the incubator. Count colonies and calculate CFU per mL.
      CFU/mL = (number of colonies) × (dilution factor)/volume plated (mL).
   b. Bacterial replication occurs during the 24 h incubation period. Routinely, we observed at least a 3-log increase in bacterial numbers between 0 h and 24 h. For representative results, please see Figure 2.
10. Day 5–10 Measure virus by plaque assay. Note that this protocol is for measurement of infectious virus by plaque assay. Other forms of measurement, including TCID 50, qRT-PCR or other methods of your choice can be used instead.
   a. The plaque assay protocol is detailed in step 6.
   b. We routinely observed higher viral replication levels in the presence of *S. aureus* with this protocol. For representative results of the difference between mono- and co-infected cultures, see Figure 3.

⚠ CRITICAL: * For host RNA extraction, you will need to pool multiple wells. We have found that 6 wells of a 12 well plate provide sufficient RNA. ** For host proteins, you will need to pool multiple wells. We have found that 6 wells of a 12 well plate provide sufficient protein for western blot analysis.

⚠ CRITICAL: When performing the assay, you MUST filter the virus only samples the same as co-infected samples. The filtration of the sample results in a decrease of PFU/mL of approx. 1-log. Therefore, for accurate comparisons to be possible, all samples need to be filtered.

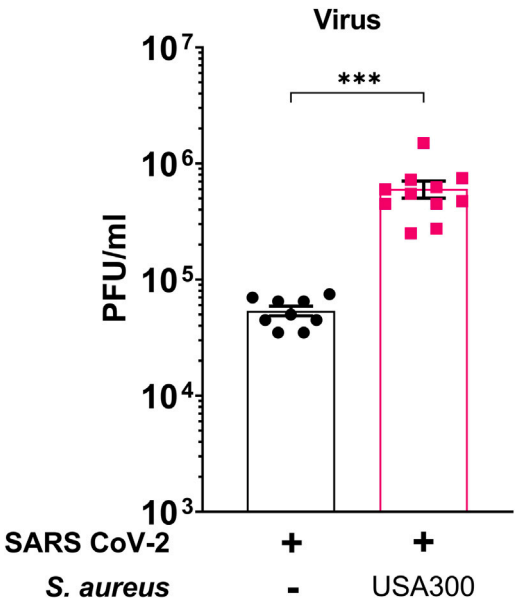

Figure 3. SARS-CoV-2 replication is enhanced in the presence of *S. aureus*

Vero E6 cells were infected with CoV-2 at MOI of 1 for 1 h and overlayed with 1 mL of serum free DMEM. 1 × 10^5 CFU of WT *S. aureus* strain USA300 were added to the culture media. 24 h later, DMEM was harvested, samples were centrifuged for 1 min at 15000 × *g* the resulting supernatant was passed through a 0.22 μm filter. Samples were serially diluted and viral titer quantified by a plaque assay. Data shown are mean ± SEM of 3–5 independent experiments. Statistical analysis was determined by an unpaired student's t test, ***$p < 0.01$, Figure reprinted with permission from Goncheva et al.[1]

EXPECTED OUTCOMES

You will be able to quantify viral and bacterial replication. Overall, we observed viral yields at approx. 10^5 PFU/mL from virus alone samples, and an average of 10-fold higher in the presence of S. aureus. Bacterial replication occurs from 0 to 24 h, and the average bacterial counts are approx. 10^8 CFU/mL. When extracting host RNA, we observed an average of 500 ng/μL yield, for a total of 50 μL (so approx. 25 μg of total RNA).

LIMITATIONS

S. aureus is toxic to host cells over time. Therefore, the protocol is limited to 24 h post addition of the bacteria, before damage to host cells begins to be observed. Although it is possible to use human cells and/or cell lines, we do know that they are more susceptible to *S. aureus* toxins and are usually damaged earlier and/or more heavily.

We have observed a pro-viral effect by *S. aureus* on SARS-CoV-2 replication when we measure viral titer by plaque assay. Results will vary if other methods of viral quantification are used. In addition, it is possible there are *S. aureus* proteins that are anti-viral. However, caution should be taken with anti-viral phenotypes to ensure that there hasn't been an increase in host cell death.

The filtering of samples does result in the loss of some virus, which necessitates the filtering of the virus only controls as well. We have not made direct comparisons but have estimated from different experiments a loss of approximately 1 log of infectious vital titer. We have not made comparisons by other methods of viral measurement.

TROUBLESHOOTING

Problem 1

Bacterial contamination in samples that should not contain bacteria (Steps 11 and 12).

Potential solution

- Take care not to move your pipette that contains bacteria over other wells.

- If problems persist, you can separate samples, so that the ones containing bacteria are on a separate 12 well plate from virus controls. If that is the case, please ensure all cells are seeded from the same solution, to ensure an equal number of cells between wells and plates.

Problem 2

Lack of bacterial replication over a period longer than 8 h (Steps 11 and 12).

Potential solution

- It is possible that some antibiotic contamination can remain when splitting flasks into 12 well plates. To eliminate this problem, maintain your cells in an antibiotic-free medium at all stages.

Problem 3

Plaque assay plates do not show a uniform layer of cells post fixing (Step 6).

Potential solution

- This is most likely a result of cells not being 100% confluent at the start of the plaque assay. Try to increase the seeding density of the cells or set up 6 well plates a day earlier.

Problem 4

Insufficient concentrations of RNA from infected cells (Step 11 – optional).

Potential solution

- It is our experience that more than one well of cells from a 12 well plate is needed for good RNA yields. Try pooling several wells together before performing the RNA isolation. We use 6 wells of a 12 well plate for an average total yield of approx. 20 μg of RNA.

Problem 5

Co-infection samples have lower PFU/mL than virus alone when using a human cell line (Step 11).

Potential solution

- *S. aureus* secretes a number of species-specific toxins that target human cells. The presence of the bacteria results in host cell toxicity over time. If you are using human derived cells, we suggest decreasing the incubation time from 24 h to 16–18 h.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David Heinrichs (deh@uwo.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets or code.

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AUTHOR CONTRIBUTIONS

M.I.G. conceived the study, performed experiments, and validated methodology. M.I.G. and D.E.H. were involved in funding acquisition. M.I.G. wrote the manuscript, and all authors edited and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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