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TITLE:

Isolation of L-form Bacteria from Urine using Filtration

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KEYWORDS:

L-forms, urine, filtration, L-form medium, L-form induction, microscopy

SUMMARY:

Here, we present a protocol for the isolation of L-form bacteria from urine, using a filtration method. Complementary methods for the preparation of L-form medium, observation of L-forms by phase-contrast microscopy and induction of L-forms under laboratory conditions are also described.

ABSTRACT:

Transition of bacteria to the L-form state is thought to play a possible role in immune evasion and bacterial persistence during treatment with cell wall-targeting antibiotics. However, isolation and handling of L-form bacteria is challenging, mainly due to their high sensitivity to changes in osmolarity. Here, we describe detailed protocols for the preparation of L-form medium, isolation of L-forms from urine using a filtration method, detection of L-forms in urine samples by phase contrast microscopy and induction of L-forms in vitro. The exact requirements for survival and growth of L-forms may vary from strain to strain. Therefore, the methods presented here are intended to act as basic guidelines for establishing L-form protocols within individual laboratories, rather than as precise instructions. The filtration method can lead to a reduction in the number of L-forms in a sample and should not be used for quantification. However, it is the only method used so far for effective separation of cell wall-deficient variants from their walled counterparts and for identification of bacterial strains, which are capable of L-form switching in patients with urinary tract infections. The filtration method has the potential to be adapted for the isolation of L-forms from patients with other categories of bacterial infections and from environmental samples.

INTRODUCTION:

Virtually all bacteria are surrounded by a structure called the cell wall. The wall is important for protection against environmental stresses, helps with regular division and gives bacteria their shape¹. However, the wall is also a target for parts of the immune system and some of the best and most used antibiotics, including penicillin^{2,3}. Despite its importance, both Gram-positive and Gram-negative bacteria can occasionally survive without the wall⁴⁻⁸. If the surrounding conditions provide enough osmoprotection to prevent them from bursting and cell wall-targeting agents are also present, bacteria can transition into a wall-less state, referred to as an L-form⁴⁻⁸.

Numerous reports indicate that switching to an L-form state and back to a walled state may be important in vivo as a mechanism for bacteria to survive both the attack from the host immune system and treatment with cell wall-targeting antibiotics⁹⁻¹⁵. Such a transition potentially provides a powerful strategy for the recurrence of a bacterial infection⁹⁻¹⁵. Understanding the basic biology of L-form bacteria and their interactions with the host are critical to decipher their role in pathogenesis. However, handling L-form bacteria is challenging.

Firstly, due to the lack of the cell wall, L-form bacteria are prone to bursting in response to changes in osmolarity. Additionally, L-forms divide in a highly irregular manner, have unpredictable patterns of growth (usually much slower than their walled counterparts) and, depending on strain, may propagate better on semi-liquid, rather than solid or liquid media. All the above considerations make quantification and comparisons of growth rates difficult. Different bacterial species (or even strains) have diverse metabolic requirements for L-form switching and growth. For example, L-forms of certain Gram-positive bacteria, which rely on aerobic respiration, are more sensitive to reactive oxygen species than their walled counterparts¹⁶.

Induction of L-forms under laboratory conditions and in the host is usually driven by cell wall-targeting agents, such as antibiotics and lysozyme⁹. Such a treatment might result in only a partial cell wall loss and therefore some walled (or partially walled) bacteria could be present in the samples, making it hard to distinguish whether any observed experimental outcomes are due to the presence of L-forms or walled forms of bacteria. The frequency of L-forms induced in vivo tend to be low, meaning they can be difficult to find and isolate. Finally, owing to their polymorphic morphology, L-forms can be easily confused in situ with structures of eukaryotic origin, such as apoptotic bodies or various granules.

Since their discovery in 1935¹⁷, several methods have been developed to handle L-forms in the laboratory. Most of these rely on the addition of an osmoprotective agent to the growth medium; usually a sugar or a salt⁹⁻¹⁸. As mentioned above, L-forms often occur side by side with walled bacteria in patient samples and separating the two populations can be difficult. However, it has been demonstrated that, unlike walled bacteria, L-forms can pass through a 0.45 μm filter due to their flexibility and variable sizes. A method using such a filter has been employed to isolate L-forms from urine^{10,19-21}.

Here, we present a protocol for the isolation of L-form bacteria from urine, using a filtration method (**Figure 1**). Complementary protocols for the preparation of L-form medium, microscopic observation of L-forms and induction of L-forms in vitro are also described.

PROTOCOL:

1. Preparation of L-form medium (LM)

1.1. Weigh out sucrose (amount adjusted to achieve a final concentration of 0.58 M), MgSO₄ (final concentration 8 mM) and Brain Heart Infusion (BHI) (amount recommended by the supplier) in a glass bottle twice the size of the desired volume of medium. To prepare 0.5 L of the medium, weigh out 100 g of sucrose, 1 g of MgSO₄ and 18.5 g of BHI and place in a 1 L glass bottle. This will allow for easier resuspension of the ingredients prior to autoclaving.

1.2. If solid or semi-solid media are required, add a desired amount of agar. For solid media, add 5 g of agar to achieve a final concentration of 1% in 0.5 L of medium. For semi-solid media, add 1 g of agar to achieve a final concentration of 0.2% in 0.5 L of medium.

1.3. Top off with deionized (DI) water to the final desired volume.

1.4. Close the bottle and mix well by shaking. There is no need to resuspend the ingredients completely, just make sure they are not clumped together at the bottom of the bottle. Loosen the cap and autoclave on a sensitive media cycle (115 °C for 15 min). Mixing the ingredients prior to autoclaving and using a sensitive media cycle is recommended to prevent clumping of the ingredients and sucrose caramelization.

1.5. Visually examine the medium following autoclaving. No clumps should be present and the medium should be amber in colour (**Figure 2A**).

1.6. Following autoclaving, let the medium cool down to a temperature which allows the bottle to be comfortably held by hand.

1.7. If the medium is required for the induction of L-forms, add an antibiotic and/or a lytic agent at this point (for example, phosphomycin at a final concentration of 400 µg/mL, penicillin G at 200 µg/mL, D-cycloserine at 400 µg/mL, ampicillin at 100 µg/mL, moenomycin at 50 µg/mL, lysozyme at 100 µg/mL or lysostaphin at 5 µg/mL). Empirically establish the type of L-form inducing agent (or agents if more than one is required) and its concentration for each of the bacterial species tested.

1.8. To prepare individual aliquots, wear gloves and work in a microbiological safety cabinet or use a Bunsen burner. The media might require incubation for prolonged periods of time and sterility is of paramount importance.

1.8.1. To prepare aliquots of solid medium, transfer 25 mL to individual 92/16 mm Petri dishes

and let them set. Using a pipettor rather than pouring plates directly is recommended to reduce the risk of contamination. Do not overdry the plates.

1.8.2. To prepare semi-liquid aliquots, transfer 5 mL of medium into multiple 30 mL polystyrene universal containers using a pipettor and let them set.

1.9. Use the media immediately or store at 4 °C for up to a week before use. Check manufacturer recommendations before storing medium containing antibiotics as the concentration might decrease, due to the compound degrading over time.

2. Isolation of L-forms from urine

2.1. Wear gloves and a lab coat during the laboratory procedures. Work in a microbiological safety cabinet or use a Bunsen burner. Wear safety goggles during filtration.

2.2. At least 1 h before the predicted arrival of the urine samples, wipe the working area with 70% ethanol and set out the required number of semi-liquid LM medium aliquots.

NOTE: A suspension of *E. coli* ST144¹⁰ L-forms induced in the laboratory in liquid LM medium, rather than human urine samples, will be used to demonstrate the protocol.

2.3. Wipe the working area again with 70% ethanol before processing the samples. To one side of the bench, lay out the desired number of 0.45 µL cut off filters, 20 mL syringes and several empty sterile 30 mL polystyrene universal containers equal to the number of samples. Add several spare filters on top of the number of the expected urine samples, just in case more than one filter will be needed to process some of the samples.

2.3.1. Place the LM medium aliquots and empty sterile 30 mL polystyrene universal containers in a stable rack in the middle of the bench. If microscopic examination is to be carried out in parallel, also prepare glass microscope slides, 22 x 22 mm coverslips, 2 µL and 1 mL pipettes, sterile 10 µL and 1 mL tips, sterile 1.5 mL tubes and make sure a bench microcentrifuge is available.

2.4. Transport urine samples from the donor to the laboratory as soon as possible following donation, to prevent potential deterioration of L-form bacteria in the sample.

2.5. Put on gloves and goggles, remove the urine samples from the safety bag, spray them with ethanol, wipe and place in the rack, near the LM medium aliquots and sterile spare 30 mL polystyrene containers. From this point, process one sample at a time.

2.6. Loosen the caps on a tube containing LM medium, spare polystyrene universal container and tube containing urine.

2.7. Remove the syringe from the package, remove the plunger and place to your right.

2.8. Pull the safety paper from the back of the filter package and attach the syringe firmly to the filter (keep the filter face down in the individual manufacturer plastic holder to make sure it remains sterile).

2.9. Working quickly but carefully, remove and discard the cap from the tube of LM medium and place the filter with the syringe on top of the tube.

2.10. Remove the cap from the urine sample and gently pour ~10 mL into the syringe (only 2 mL will be filtered but excess volume prevents splashing), making sure over 1 mL is left for a microscopic examination. Only 0.5 mL will be used, but it is impractical to precisely measure such a volume while performing filtration, therefore an approximate excess volume is retained.

2.11. Very carefully place the plunger into the back of the syringe. A small amount of resistance can be felt before the plunger is placed in a fully operational position and it is easy to spill the sample past this point. Practice several times using water before processing urine samples.

2.12. Gently press the plunger and pass 2 mL of urine through the filter until an increasing resistance is felt. Be careful not to apply too much pressure to prevent the sample from spilling over and the filter from breaking. If the sample is particularly dense and generates too much resistance, pass the sample through multiple filters, rather than forcing it through one.

2.13. Gently tap the filter against the tube containing LM media to dislodge any remaining filtered sample, lift the filter and the syringe and quickly place the cap from the spare 30 mL polystyrene universal container on the tube containing LM medium and filtered urine, making sure not to touch the inside of the tube with the edge of the cap. Safely dispose of the filter, syringe and spare polystyrene container.

2.14. Incubate the samples in a stationary position at 30 °C for up to 1 month. This will allow L-forms that passed through the filter to regenerate their walls and start growing as walled bacteria.

2.15. Observe the samples every day for evidence of growth, holding the tubes containing filtered samples against a light source. For positive samples, the growth usually appears within 3 to 7 days.

2.16. Once the growth has been detected, transfer the sample to a microbiological safety cabinet or within proximity of a Bunsen burner. Open the sample and carefully dip in a 5 µL plastic loop, aiming for the area of growth, and then streak the samples on standard solid BHI medium without osmoprotection, aiming to obtain single colonies²².

2.17. At the same time, examine a small amount of the sample, together with a fraction of semi-liquid medium (~5 µL), microscopically as described in step 3. Transferring the sample onto a microscopic slide together with the medium will ensure that any L-forms which may still be

present in the sample will not burst and can be detected. However, the majority of the bacteria at this point are expected to have reverted to walled forms and appear as regular rods or cocci.

2.18. Incubate the samples streaked on the BHI plates in a stationary position at 37 °C overnight.

2.19. Examine for evidence of growth, pick a single colony from each plate using a 5 µL loop and inoculate into 5 mL of liquid BHI. Incubate at 37 °C overnight, with shaking.

2.20. Use the overnight cultures to prepare glycerol stocks or to isolate DNA for species identification using a preferred method (e.g., Qiagen DNeasy Blood & Tissue Kit).

3. Examination of urine samples for the presence of L-forms by phase contrast microscopy

3.1. Place the remaining 0.5 mL of urine sample in a microcentrifuge tube and spin at 8,000 x g for 1 min in a bench microcentrifuge.

3.2. Gently remove the supernatant, leaving behind approximately 20 µL and resuspend the pellet by pipetting gently 3 times up and down.

3.3. Place 1 µL onto a glass microscope slide and cover with a 22 x 22 mm coverslip (to examine a 5 µL sample (see step 2.17) use a 22 x 50 mm coverslip to make sure the coverslip will adhere well to the slide). Press down on the coverslip gently using a sterile cotton wool pad to create a seal and to check that the cover slip is not able to move. Discard the cotton wool pad.

3.4. Examine the slide using a microscope equipped with a 100x phase contrast objective for the presence of L-form-like structures (**Figure 3**).

NOTE: To confirm bacterial origin of L-form-like structures in patient samples, it is recommended to examine the samples by fluorescent in situ hybridisation (FISH) using oligonucleotide probes specific for bacterial sequences^{10,23}.

4. Induction of L-forms in vitro

4.1. Streak walled bacteria of choice to single colonies²² on a plate containing non-osmoprotective medium (for example BHI) and incubate stationary at 37 °C overnight.

4.2. The next day, prepare osmoprotective LM medium, containing L-form inducing agent/s of choice as described in step 1. Prepare the desired number of plates with solid medium.

4.3. Using a sterile 5 µL plastic loop or a toothpick, pick a generous amount (~4-5 colonies worth) from the non-osmoprotective BHI plate incubated the night before.

4.4. Streak bacteria on the LM plate using the flat surface of the loop (rather than the edge).

Streak the bacteria with one continuous motion on the edge of the plate, then turn the plate 90°. Touch the loop to the edge of the first streak and with a continuous motion, spread the streak over a quadrant of the plate, gradually diluting the bacteria with multiple overlapping streaks (the whole plate can be used if desired. If using only a quadrant of the plate, other quadrants can be used for multiple inductions).

NOTE: **Figure 4A** shows schematically the direction of streaking. The aim here is to obtain an area of growth containing as many pure L-forms as possible, rather than single colonies. For that reason, there is no need to change the loops between streaks. Single colonies of L-forms can be very difficult to obtain and using too small a number of bacteria could result in no L-form growth at all. On the other hand, if too many walled bacteria have been used for the induction, not all bacteria will undergo the switch and some may still retain the wall. The best L-form growth area can usually be detected in the middle of the streak.

4.5. Place the plate in a plastic bag or other container to prevent it from drying and incubate at 30 °C (L-forms of some bacterial species might grow better in anaerobic conditions, so an anaerobic chamber can be tested for more efficient L-form growth). Examine the plate for evidence of growth every day. This usually appears within 3 to 7 days. The amount of growth is strain specific. An example of L-form growth is shown in **Figure 4B**.

4.6. Confirm the presence of L-forms using a microscope. Place a 2 µL droplet of LM medium on a microscopic slide and, using a pipette tip, pick a small amount of cells from the middle of the streak and resuspend in the droplet.

4.6.1. Cover with a 22 x 22 mm coverslip and examine for the presence of L-forms using a microscope with a 100x phase contrast objective. A representative example can be found in **Figure 4C**.

REPRESENTATIVE RESULTS:

L-form media containing sucrose can undergo varying degrees of caramelization following autoclaving, which are associated with a change in color. **Figure 2** shows representative outcomes of autoclaving media containing sucrose. **Figure 2A** shows a typical example of LM medium, which has turned amber in color following the autoclaving. **Figure 2B** shows a typical example of 1.16 M sucrose solution (2 x concentration required for making LM medium) following autoclaving. Occasionally, LM medium or sucrose may undergo extensive caramelization during autoclaving, and it is not recommended to use the medium if this happens. **Figure 2C** shows an example of over-caramelized sucrose medium.

L-form bacteria can be highly heterogeneous and **Figure 3** shows examples of L-form-like structures observable in patient urine samples. To confirm the bacterial origin of the L-form-like structures found in urine samples, FISH with fluorescent probes targeting bacterial sequences is recommended^{10,23}.

Growth levels of L-forms induced under laboratory conditions are strain specific. **Figure 4B** shows an example of *Bacillus subtilis* L-form growth and **Figure 4C** shows the microscopic appearance of L-forms induced in **Figure 4B**.

FIGURE AND TABLE LEGENDS:

Figure 1: Isolation of L-forms using filtration method – schematic representation. Urine sample is passed through a 0.45 µm filter into a polystyrene universal container, containing osmoprotective LM medium supplemented with 0.2% agar. The sample is then incubated in a stationary position at 30 °C for up to a month and visually checked daily for evidence of growth. This incubation period allows any L-forms that are present in the sample to regenerate their cell walls. Once the growth is detected, the bacteria can be re-streaked on a plate containing regular, solid, non-osmoprotective medium (such as nutrient agar or brain-heart infusion) to isolate single colonies, which can then be subjected to DNA extraction and sequencing to identify the isolated bacterial species.

Figure 2: L-form media. (A) LM medium following autoclaving. (B) 1.16 M sucrose (2 x concentration) following autoclaving. (C) Extensively caramelized LM medium.

Figure 3: Examples of L-form like structures observable in urine of patients with recurrent UTI by phase contrast microscopy. (A,B) Structures typical of dividing bacterial L-forms suspended in urine. (C,D) Structures typical of dividing bacterial L-forms associated with eukaryotic cells. (E,F) Crescent shaped structure characteristic for Gram-negative L-forms (red arrow). (F,G) Structures typical for intermediate stages of transition between the walled cells and L-forms (red arrow in G). (H) Intracellular vesicles typical for large L-forms. Scale bar = 5 µm. This figure has been modified from¹⁰.

Figure 4: Streak-plate technique for induction of L-forms on solid media. (A) Schematic representation of streaking bacteria using a quarter of a plate for induction of L-forms. The arrow indicates the direction to streak the bacteria. (B) An example of *Bacillus subtilis* L-form growth following streaking, as shown in (A), after 3 days incubation at 30 °C. (C) L-forms induced in (B) visualized by phase contrast microscopy. Scale bar = 5 µm. Panels B and C have been modified from¹⁶

DISCUSSION:

Protocols described in this manuscript have been used to isolate and handle L-forms of various bacterial species from human urine, including *E. coli*, *Streptococcus*, *Staphylococcus*, *Klebsiella*, *Pseudomonas*, *Enterococcus* and *Enterobacter spp*, all of which are typically associated with UTIs^{10,24}. However, methods for handling L-forms, established in one laboratory may not work immediately in another and what works for one bacterial strain may not work for another. Therefore, the protocols described here are likely to require multiple attempts and additional optimization. In particular, nutrient requirements, oxygen availability and fluidity of the medium may need to be tested. As well as the obvious requirement for osmoprotection, conditions optimal for L-form transition and growth could be different from conditions optimal for growth

of walled forms of the same species. Moreover, several technical issues could be encountered when attempting the protocols.

A common problem associated with preparation of L-form media is sucrose caramelization following autoclaving. If the medium turns dark brown in color, it should be discarded and a fresh batch should be prepared. It may be necessary to prepare the sucrose in water in one bottle and the remaining ingredients in another, both at 2x concentration. 2x concentrated solutions can then be combined at a 1:1 ratio following autoclaving, to achieve the desired final concentrations. Sucrose autoclaved separately may turn slightly yellow in color (**Figure 2B**), which is acceptable, but if it turns dark brown (**Figure 2C**), it should not be used for experiments. Adjusting the length and the temperature of the autoclaving cycle might be necessary to alleviate sucrose caramelization. Testing sterility of the media prepared using an adjusted autoclave cycle, by incubating an aliquot of each of the media for at least 3 days at 37 °C, is recommended. Finally, it might be necessary to test an alternative osmoprotective agent, such as salt, if the problem with caramelization persists¹⁸.

Several issues might also be encountered during isolation of L-forms from patient samples. As mentioned in the protocol, L-forms present in the samples might potentially deteriorate; therefore, it is important to transport the samples to the laboratory as soon as possible after donation. For the same reason, storing samples at low temperatures or modifying sample composition (for example by addition of PBS) is strongly discouraged.

The filtration method itself has its limitations. Some L-forms may be ripped apart due to shear forces generated by the flow of media through the filter. In the study by Mickiewicz et al., only 41% of L-forms in control samples, which contained laboratory induced *E. coli* L-forms, passed through the filter¹⁰. This demonstrates that the filtration method can lead to an underestimation of the number of positive samples and is not recommended for quantitative studies.

On very rare occasions, following filtration, a significant growth might be observable the next day in the semi-liquid media aliquots used for L-form isolation. This could be an indication that either the filter broke during the protocol, allowing the passage of walled bacteria, or the sample was contaminated. It is good practice to discard such samples or at least treat them with caution. Daily visual examination of the samples is critical to prevent false positives. Before processing urine samples by filtration, it is recommended to pass several samples of laboratory induced L-forms, walled bacteria and sterile medium through the filter of choice, to control for efficiency of L-form separation, potential passage of walled bacteria due to filter breakage and to make sure the sterile technique used is working well.

In rare cases, stable L-forms might be isolated by filtration, with no walled forms detectable by microscopy. To maintain viable L-form bacteria, it is recommended to transfer several “loop-fulls” of the sample to a tube containing fresh semi-liquid LM medium or to re-streak on solid osmoprotective medium every 3-7 days, depending on efficiency of the growth. If no walled forms appear after several passages, freezing samples in 40% glycerol at -80 °C could be

attempted to preserve the isolate; however, some L-forms may not tolerate such a procedure well.

Despite its limitations, the filtration method is the only one used so far for the separation of L-forms from walled forms in patient samples. It allows for the identification of bacterial strains capable of L-form switching in vivo. There is the potential to develop and adapt the filtration method for isolation of L-forms from other types of human or environmental samples (for example blood or plants).

Taking into account all of the above considerations, we recommend the protocols outlined in this manuscript as a good starting point for developing tailored L-form protocols in individual laboratories, rather than as rigid instructions. Working with L-forms requires great care, dedication and patience but with practice, it can be extremely rewarding. We hope that the guidelines outlined in this manuscript will become a benchmark for developing L-form protocols and will encourage more basic and clinical research groups to investigate these fascinating bacterial forms.

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DISCLOSURES:

The authors have nothing to disclose.

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Name of Material/ Equipment	Company	Catalog Number
0.45 µL cut off filters	Sarstedt	83.1826
20 mL syringes	Fisher	17955460
30 mL polystyrene universal tubes	Starlab	E1412-3010
92 x 16 mm Petri Dishes	Starstedt	82.1473
Agar	Oxoid	LP0011
Ampicillin	Sigma-Aldrich	A9518
Brain Heart Infusion	Oxoid	CM1135
Cover slips (22 x 22 mm, 22 x 50 mm)	VWR	631-0137/-0125
D-cycloserine	Sigma-Aldrich	C6880
Glass microscope slides	VWR	631-1550P
Lysostaphin	Sigma-Aldrich	L7386
Lysozyme	Sigma-Aldrich	L4919
MgSO ₄	VWR	25165.26
Moenomycin	Sigma-Aldrich	32404
Penicillin G	Sigma-Aldrich	13752
Phosphomycin	Sigma-Aldrich	P5396
Sucrose	Sigma-Aldrich	84100

Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been revised for spelling or grammar issues.

2. Keywords: Please provide at least 6 keywords or phrases.

Provided.

3. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10–50 words: "Here, we present a protocol to ..."

Provided.

4. Abstract: Please also include a summary of the advantages, limitations, and applications of the protocol.

Abstract revised.

5. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

No human samples will be used for the demonstration of the protocol. Point 2.1 specifies that laboratory induced L-forms will be used instead of human urine samples to demonstrate the protocol.

6. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

Revised.

7. 1.1: Please list an approximate volume of media to prepare. Please specify the amount of BHI needed here.

The amount of media will be dependent on the individual demand of the person performing the experiment and providing final concentrations required rather than volumes allows precise weighing of the desired ingredients. However, we have now included an example required for 0.5 l volume of media.

8. 1.6.1: How large is the Petri dish?

Information included in the protocol text and in the Excel spreadsheet.

9. 3.1, etc.: Please list all centrifugation speeds in terms of centrifugal g-force (x g) instead of rpm.

RPM converted to g-force

10. Please reference each panel of Figure 3 in the manuscript text.

The panels have been referenced.

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12. References: Please do not abbreviate journal titles; use full journal name.

Full journal names included.

13. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

Information provided and the materials sorted alphabetically

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes a protocol to isolate cell wall deficient bacteria (known historically as L-forms). The authors note that pathogenic bacteria could potentially evade the host immune system and become resistant to some classes of antibiotic by transitioning to an L-form-like state. There were tantalising hints in the literature from the 1960s and 70s that this was the case. However, definitive proof was missing partly because of a lack of reliable and reproducible protocols for their isolation and study. This was the case until the publication of a paper in Nature Communications (Mickiewicz et al. (2019) <https://doi.org/10.1038/s41467-019-12359-3>) that demonstrated the isolation and identification (using modern techniques) of L-form-like bacteria from the urine of patients with recurrent urinary tract infections. The current manuscript described protocols for such isolation in more detail than the Nature Communications paper. It is, therefore, an important publication because it sets the standard for the L-form field in academia and in the clinic (as noted by the authors).

Major Concerns:

Poor sterile technique in step 2.6 (line 143). The authors state:-

"Working quickly, remove the cap from LM media, place it face down on a bench and place the filter with the syringe on top of the container with the media."

Perviously in the protocol it is also stated (line 115):-

"Wipe the bench with 70% ethanol and set out the required number of semi-liquid LM media aliquots (place the media on the bench at least 1 h before the predicted arrival of the urine samples)."

- Bacterial spores are resistant to ethanol (Russell (1990) Clinical Microbiology Reviews: 3(2); 99-119).

- Cleaning the bench 1 hour before the preparation of samples further allows contaminated dust particles to fall on its surface. This is the case unless working in a microbiological safety cabinet or if a burner is left running for this one hour period. Neither of which is called for in section 2 nor is it safe practice to leave a burner running unattended.

- Also, bacteria can be transferred to the bench surface by the hand during sample preparation.

For these three reasons, I would consider the bench surface not to be sterile in the conditions described. It is therefore inadvisable to place a cap face down on this surface.

Standard microbiology technique is to keep this cap in the crook of your hand. If is not possible to do this during the filtering process, I would advise the authors to discard the cap, perform the filtering, then close the tube with a cap from another tube kept in a sterile package.

Reviewer suggestions have been included in the protocol.

As the urine samples are opened, aerosols can transfer bacteria in droplets of media to the bench surface. The cap can then transfer such bacteria to the rim of the tube of the LM media aliquot. Incubation for periods up to one month would allow condensation to form inside the tube and the bacteria to be drawn down to the surface of the agar. While I do admit that such a situation is unlikely, it is possible that poor sterile technique could allow bacteria to bypass the filter. Indeed, paragraph 320-324 acknowledges the occurrence of false positives. While the author ascribes this to a broken filter, such instances could instead arise from poor sterile technique. I am not saying that this certainly happened, but I would like some acknowledgement of this so that the reader can decide for themselves to how frequently this might happen.

The possibility of sample contamination has been acknowledged.

While it is plausible that the lack of a cell wall would allow L-forms to pass through a 0.45 μm filter, I would like to see an acknowledgement of an alternative hypothesis, namely that shear forces generated by the flow of media through the filter would rip apart L-forms despite their flexible envelopes. To make a proper judgement about which scenario is true, I would like to see data on the false positive rate that compared to the true positive rate.

This is a very important consideration, which has now been acknowledged both in the abstract and in the discussion.

Also, is it possible to perform experiments on walled bacteria to determine how often the filter breaks? Finally, the failures in sterile technique necessitate a control experiment where sterile media is processed exactly as describe here (including handling of contaminated samples in the same working area) to determine the rate at which placing a lid on surface which is not sterile would lead to bacteria growing in the LM media without passing through the filter. Only then can the reader judge if the risk of contamination is sufficiently low enough.

Suggestions to include the control experiments listed by the reviewer have now been included in the discussion.

One further failure in good laboratory practice occurs in step 3.3 (line 201):-

"Press the coverslip gently to create a seal. Check with your finger that the cover slip is not able to move, ensuring not to press too hard to avoid cracking the glass"

Such an operation should not be performed with samples from patients that almost certainly contain pathogenic bacteria when the potential exists to spread bacteria to other surfaces with a gloved hand or for sharp glass edges to cut the skin through the glove. Stating that the reader should "not press too hard" is insufficient.

This section has been revised and use of cotton wool pad has been suggested to prevent direct contact with the glove and potentially cutting the glove.

Minor Concerns:

The manuscript is sloppily prepared and poorly written throughout. For example, there are formatting errors (there was highlighted text throughout the pdf that I received), typing errors (BHA instead of BHI, line 186), inappropriate references (references 2 and 3, line 38, do not support the preceding statement while the next sentence has no references, line 40).

Also there are grammatical errors and opportunities for stylistic improvements throughout the manuscript (too numerous to list). For example, in the first sentence of the abstract (line 26) should be reworded to make it clear that it is the "transition" of bacteria to the L-form state that allows them to evade the immune system.

The errors pointed out by the reviewer have been corrected and the manuscript has been improved throughout.

Reviewer #2:

Manuscript Summary:

This manuscript provides some basic information to work with fragile bacterial variants called L-forms. The methods have been described well, and I only have a few suggestions to clarify some terminology.

Major Concerns:

None

Minor Concerns:

Questions/remarks and typos

LM media is plural. Is this OK? I would use LM medium in the text.

Media changed to medium throughout the manuscript, where appropriate

Line 54; "for L-form switching..."

Corrected

Line 69: "...a 0.45 uM filter"

Corrected

Line 86: "what containers are used? This seems a broad term. Do the authors mean Falcon tubes?"

The manufacturer refers to the tubes as "polystyrene universal containers" and we now retained this term throughout the protocol. Additionally, serial number was listed in the Table of Materials.

Line 87: What id "DI water"?

Deionised water (corrected)

Line 98: "...concentration of 200..."

Corrected

Line 101/102: "...has to be established ..."

Corrected

Line 103: "...or use a Bunsen..."

Corrected

Line 119: "Set aside the desired number..."

Corrected

Line 119: The two spare filters seems arbitrary and surely depends on the number of urine samples that will be filtered. With 10s of urine samples I am sure more spare filters are needed

Changed to several

Line 135: rephrase (I think the authors loosen the caps of the tube with LM medium and the tube with the urine?)

Rephrased

Line 137: "open the syringe package, ..." (the syringe is still in the package)

Rephrased

Line 140: "...make sure it remains sterile"

Corrected

Line 143: "...remove the cap from the tube/container with LM media"

Rephrased

Sections 2.7-2.8: it seems that the amount of filtrate is not very precise (approx. 2 ml, approx 10 ml etc). In this manner the concentration of medium varies from experiment to experiment?

Precise volumes have been provided where possible. It has been explained in the protocol why in few places exact measurements were not practical to perform.

Line 160: What is "the universal"? Please rephrase (or use the work tube/container or something)

The manufacturer refers to the tubes as Polystyrene Universal Containers and we retained this term throughout the protocol. Additionally, serial number was listed in the Table of Materials.

Line 173: Introduce "BHI"

BHI introduced in the section 1.1

Line 180: "... expected to have reverted..."

Corrected

Line 186: BHA?

Corrected to BHI

Line 193: does centrifugation at 13,000 rpm not kill L-forms?

In our experience centrifugation does not kill L-forms, however we have now reduced the speed recommended in the protocol to accommodate for such a possibility.

Line 223: "...schematically the direction of streaking..."

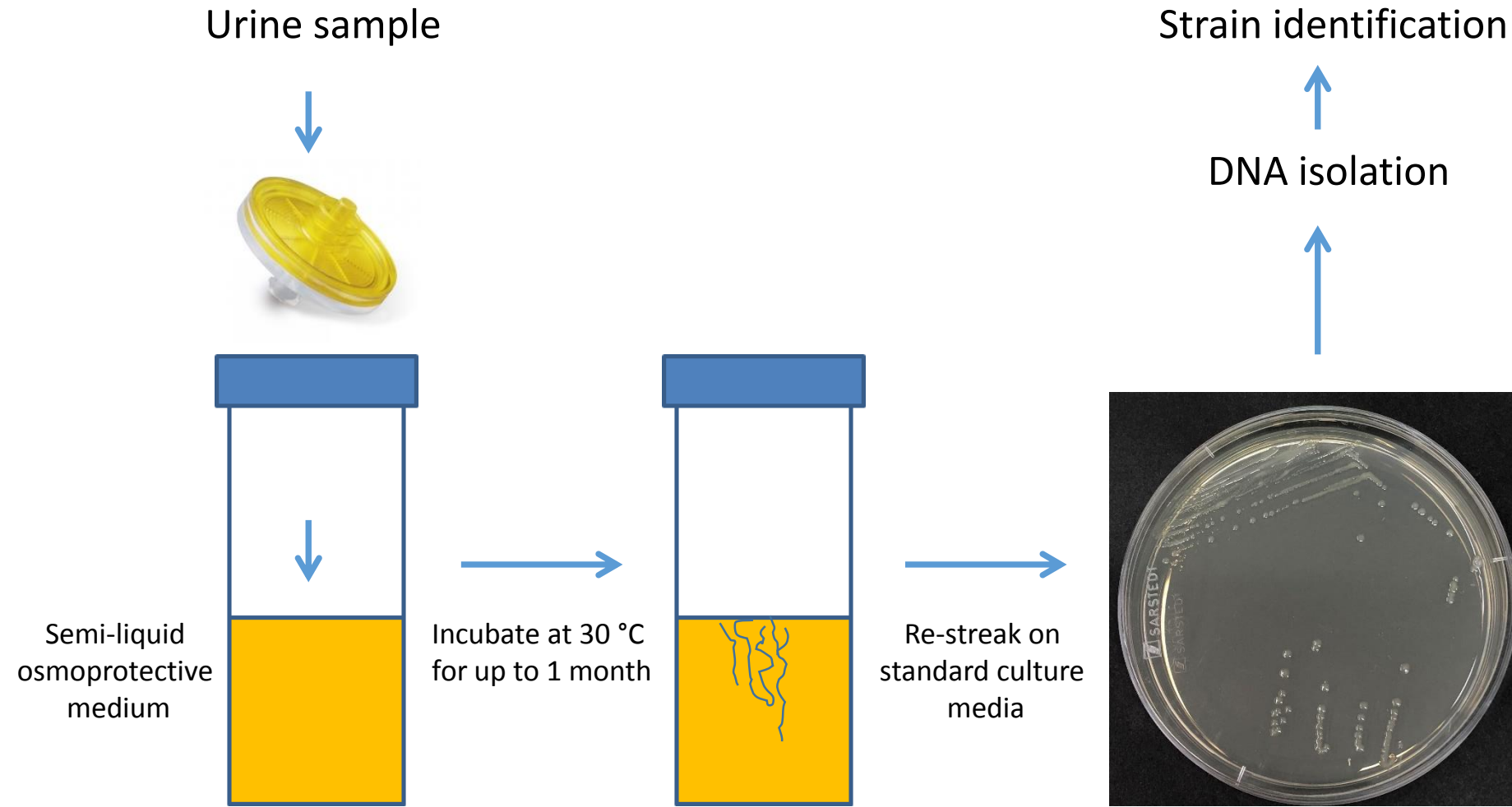
Corrected

Figure 2: why is the bottle with 2x sucrose in panel B less dark than that of panel A?

Panel A shows LM medium, which contains sucrose and BHI and panel B shows sucrose alone

Line 314: if salt is used, what salt is used and how much is used? Also, salt (NaCl) in water ends up as ions, which present not only osmotic strength but also charge. Is there no effect?

We do not tend to use salt in our laboratory and have not yet investigated the effects of salt on L-forms. We have included a reference, where another research group have used NaCl for osmoprotection of L-forms.



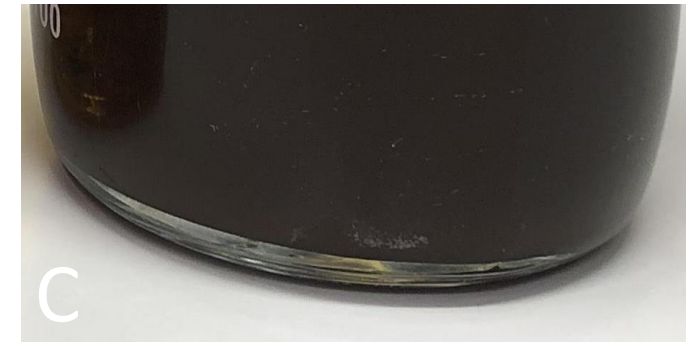
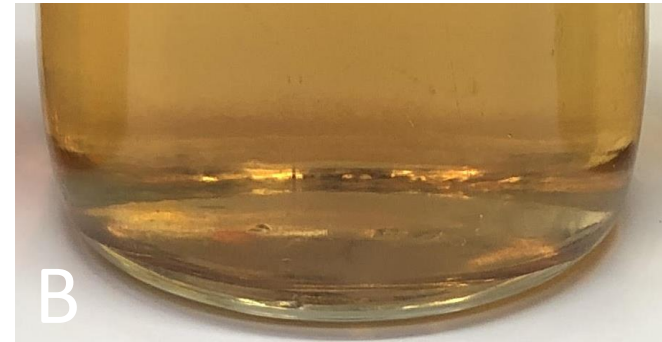
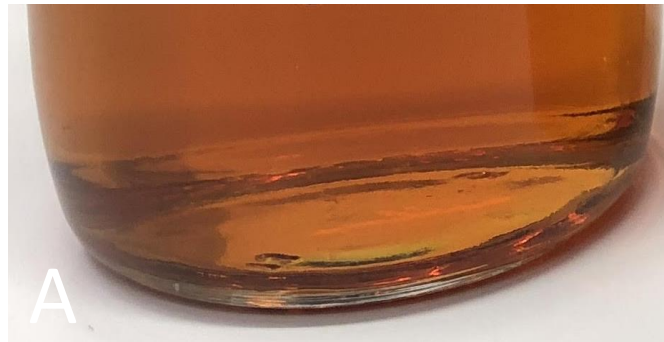


Figure 3

[Click here to access/download;Supplemental File \(Figures, Permissions, etc.\);Figure 3.pptx](#) 

