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Combination of Adhesive-tape-based Sampling and Fluorescence in situ Hybridization for Rapid Detection of Salmonella on Fresh Produce

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Abstract

This protocol describes a simple approach for adhesive-tape-based sampling of tomato and other fresh produce surfaces, followed by on-tape fluorescence in situ hybridization (FISH) for rapid culture-independent detection of Salmonella spp. Cell-charged tapes can also be placed facedown on selective agar for solid-phase enrichment prior to detection. Alternatively, low-volume liquid enrichments (liquid surface miniculture) can be performed on the surface of the tape in non-selective broth, followed by FISH and analysis via flow cytometry. To begin, sterile adhesive tape is brought into contact with fresh produce, gentle pressure is applied, and the tape is removed, physically extracting microbes present on these surfaces. Tapes are mounted sticky-side up onto glass microscope slides and the sampled cells are fixed with 10% formalin (30 min) and dehydrated using a graded ethanol series (50, 80, and 95%; 3 min each concentration). Next, cell-charged tapes are spotted with buffer containing a Salmonella-targeted DNA probe cocktail and hybridized for 15 - 30 min at 55°C, followed by a brief rinse in a washing buffer to remove unbound probe. Adherent, FISH-labeled cells are then counterstained with the DNA dye 4',6-diamidino-2-phenylindole (DAPI) and results are viewed using fluorescence microscopy. For solid-phase enrichment, cell-charged tapes are placed face-down on a suitable selective agar surface and incubated to allow in situ growth of Salmonella microcolonies, followed by FISH and microscopy as described above. For liquid surface miniculture, cell-charged tapes are placed sticky side up and a silicone perfusion chamber is applied so that the tape and microscope slide form the bottom of a water-tight chamber into which a small volume (≤ 500 μL) of Trypticase Soy Broth (TSB) is introduced. The inlet ports are sealed and the chambers are incubated at 35 - 37°C, allowing growth-based amplification of tape-extracted microbes. Following incubation, inlet ports are unsealed, cells are detached and mixed with vigorous back and forth pipetting, harvested via centrifugation and fixed in 10% neutral buffered formalin. Finally, samples are hybridized and examined via flow cytometry to reveal the presence of Salmonella spp. As described here, our "tape-FISH" approach can provide simple and rapid sampling and detection of Salmonella on tomato surfaces. We have also used this approach for sampling other types of fresh produce, including spinach and jalapeño peppers.

Keywords

Immunology, Issue 44, Salmonella, adhesive tape, rapid detection, fresh produce, fluorescence in situ hybridization, fluorescence microscopy, flow cytometry

Disciplines

Food Science | Human and Clinical Nutrition

Comments

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This article includes a video component, which may be found at http://www.jove.com/video/2308/.

Video Article

Combination of Adhesive-tape-based Sampling and Fluorescence *in situ* Hybridization for Rapid Detection of *Salmonella* on Fresh Produce

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Video Link

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Protocol

1. Surface Sampling with Sterile Adhesive Tape

1. Select a tape to use for sampling. The commercially available Fungi-Tape or Con-Tact-It sampling tapes are sterile and specially packaged for ease of use. However, we have found that transparent (optically clear) generic office tape can also be used.
2. Use a permanent marker to draw 1 cm^2 squares on the non-sticky side of a 10 cm piece of adhesive tape (a paper template can be used). This will serve as a visual guide for noting which portion of the tape has been used to sample the food or environmental surface.
3. Form a "C"-shaped loop of tape, with the sticky side facing the surface to be sampled. To do this, hold the sticky ends with the thumb and middle finger and position the index finger against the drawn square on the back (non-sticky side) of the tape (Figure 1).
4. Place the tape on the surface to be sampled and gently press the marked area against the surface. Without releasing the edges of the tape, use the index finger to ensure that the sticky side of the tape comes in full contact with the sample surface, avoiding bubbles.
5. Using an even motion, slowly pull the tape away from the sample, physically extracting surface-bound microbes. Fasten the cell-charged tape, sticky side up, onto a glass microscope slide using generic transparent office tape. Ensure proper tension/stretching of the tape so that a flat, non-wrinkly surface is created. This will help minimize problems with deformation/curling of the tape during subsequent heating during hybridization.

2. Solid-phase Enrichment and Liquid Surface Miniculture

1. Solid-phase enrichment is performed by placing the tape face-down on xylose-lysine-Tergitol 4 (XLT-4) agar plates so that the sampled cells are placed in direct contact with the agar surface. The templated/sample contact portion of the tape is placed flush with the agar surface and one end of the tape is loosely adhered to the side wall of the Petri dish to facilitate easy removal of the tape from the agar surface following enrichment.
2. Plates are inverted (to avoid condensation) and incubated at 35 - 37°C to allow sufficient growth of *Salmonella* spp. The length of the incubation period needed to enrich the cells to a detectable level will depend on initial *Salmonella* contamination levels. We have observed excellent microcolony formation from low initial inocula after 8 h enrichment.
3. After the desired enrichment period, open the agar plate. The tape will retain its tackiness during incubation. Gently press the tape against the agar using the index finger to ensure maximum recovery of microcolonies formed at the tape-agar interface. Grasp the tape from the edge attached to the wall of the Petri dish and remove it with a slow, even motion. Mount the cell-charged tape, sticky side up, onto a microscope slide as described in section 1.5 above. Proceed to "Fixation and Dehydration" step below.
4. For liquid surface miniculture, begin by fastening the tape used for sampling onto a microscope slide as described in section 1.5 above. Next, cover the templated/sample contact portion of the tape with a non-sterile silicone perfusion chamber (Coverwell, Grace Bio-Labs, Inc.), forming a sealed chamber whose bottom is comprised of the cell-charged tape, facing upwards. Firmly, but gently press the chamber into place to ensure a watertight seal.
5. Using a flexible gel loading pipette tip, transfer ≤ 500 µL Trypticase Soy Broth or other suitable enrichment medium through one of the chamber's small inlet ports. Seal both ports with transparent office tape to prevent evaporation and incubate slides at 35 -37°C as needed for sufficient enrichment. Although all operations should be performed according to good sampling and handling practices, sterility of port-sealing tape or perfusion chambers is not required during liquid surface miniculture. The combination of FISH and flow cytometry will enable clear discrimination of *Salmonella* spp. from non-target bacteria that may be present, with the largest contribution of non-target flora expected to come from the sample itself. Proceed to "Fixation and Dehydration" step below.

3. Fixation and Dehydration

1. For direct surface sampling or for solid phase enrichment of *Salmonella* spp. on XLT-4 agar, perform on-tape cell fixation for 30 min at 25°C by covering the sample contact area with 500 µL 10% neutral buffered formalin.
2. Discard fixative into a sealable container (under a chemical hood to minimize exposure to irritating/toxic vapor).
3. Dehydrate in an ethanol series (50%, 80% and 95%; 300 µL/3 min each concentration). Proceed to "Hybridization" step below.
4. For fixation of 500 µL liquid surface miniculture samples, perfusion chambers are unsealed, and a flexible gel-loading pipette tip is used to extract the enrichate. Rapid up and down pipetting is used to help ensure effective removal of any remaining tape-bound cells.
5. Next, the entire 500 µL miniculture volume is transferred to a microcentrifuge tube and spun down at 2,000 x g (5 min). The supernatant is discarded, the pellet is vortexed vigorously for 30-60 s, then resuspended in an equal volume of 10% neutral buffered formalin. Cells are fixed for 30 min at 25°C.
6. Next, fixative is removed and the sample is resuspended in cell storage buffer, as follows. The sample is spun down at 2,000 x g (5 min, 25°C) the supernatant is discarded, the pellet is vortexed vigorously for 30 - 60 s, the resuspended in an equal volume of 50% non-denatured ethanol-50% phosphate-buffered saline. Fixed cells can be stored indefinitely (years) at -20°C. Note: Whenever liquids are changed (for example, when introducing a fixative or different buffer), it is important to thoroughly resuspend (with vortexing) the cell pellet in a minimal portion (~10 - 20 µL) of the "outgoing" liquid system. This will help prevent clumping and ensure that even suspensions of individual cells are obtained. Proceed to "Hybridization" step below.

4. Hybridization

1. Prepare hybridization/washing buffer using the commercial molecular biology-grade solutions noted in Materials Table. Final buffer component concentrations are 0.7 M NaCl, 0.1 M TRIS [pH 8.0], 10 mM EDTA, 0.1% sodium dodecyl sulfate, made up to the final desired volume with molecular-grade water and filtered using a 0.22 µm syringe or cup filter. The same buffer, without the addition of probes (see below) is used as a washing buffer. Preheat hybridization and washing buffers to 55°C.
2. Add fluorescently-labeled Sal3 (Nordentoft *et al.*, 1997; 5'- AAT CAC TTC ACC TAC GTG-3') and Salm-63 (Kutter *et al.*, 2006; 5'-TCG ACT GAC TTC AGC TCC-3') oligonucleotide probes (Materials Table) to preheated hybridization buffer. A total probe concentration of 5 ng μL^{-1} is used (e.g. 2.5 ng μL^{-1} each probe; Bisha and Brehm-Stecher, 2009a).
3. For direct-to-tape and solid phase enrichment samples, overlay the templated sample contact area of the tape with 300 µL of hybridization buffer containing the probe cocktail and hybridize tape-bound cells in a moist, sealed incubation chamber set to 55°C. If a direct-contact incubator such as the Slide Moat instrument (Materials Table) is used, samples are hybridized for 15 min and >20 slides can be processed simultaneously. If a rotary-style hybridization oven such as the Bambino instrument (Materials Table) is used, slides are placed in 50 mL polypropylene centrifuge tubes for hybridization, one slide per tube. Because heat transfer is not direct, these samples are hybridized for longer periods (up to 30 min).
4. Following hybridization, the slides are removed and the probe-containing hybridization buffer overlay is discarded. Slides are then either briefly and gently rinsed with preheated washing buffer by pipetting a small volume of buffer over a tilted slide (3 rinses of 300 µL each), or formally washed (up to 30 min) with either an overlay of preheated washing buffer or immersion in a 50 mL polypropylene centrifuge tube containing preheated washing buffer. In our experience, although a formal wash will provide improved results (less haze from unbound probe), a simple rinse is adequate for unambiguous detection of *Salmonella* spp. Next, slides are air-dried before moving to the "Detection" step below.
5. Hybridization of liquid surface miniculture samples is performed by spinning down samples (freshly-fixed or kept in storage buffer at -20°C) for 5 min at 2,000 x g, followed by vigorous vortexing of the pellet and resuspension in 100 µL preheated hybridization buffer containing the probe cocktail. Samples are hybridized at 55°C for 30 min on a heat block or other suitable incubation station (Materials Table), followed by addition of 500 µL preheated washing buffer. As with tape-bound samples, these samples may be formally washed for up to 30 min with

further incubation at 55°C in this wash buffer, with intermittent vortexing. Alternatively, samples may be thoroughly vortexed after addition of 500 µL preheated washing buffer, followed by immediate harvesting for analysis (below).

6. In preparation for detection of *Salmonella* spp. via flow cytometry, liquid surface miniculture samples are spun down at 2,000 x g for 5 min, the supernatant is discarded and the samples are resuspended in 300 µL room temperature (~25°C) PBS. If samples require transport to a distant facility, or if a delay is expected in between hybridization and analysis, samples may be refrigerated or held on ice prior to analysis. Alternatively, samples may be transferred to cell storage buffer (50:50 mixture of PBS and absolute ethanol) and held at -20°C for up to a week without appreciable losses in probe-conferred fluorescence (Bisha and Brehm-Stecher, 2009b).

5. Detection

1. For on-tape detection of *Salmonella* spp. via fluorescence microscopy, direct-to-tape or solid phase enrichment samples are overlayed with ~10 µL Vectashield H-1200 mounting medium containing the nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI), mounted with a cover slip, then incubated in the dark for 10 min.
2. Immersion oil is placed on the cover slip, and samples are examined using a high-magnification oil objective (63x or 100x). The DAPI filter is used to bring the sample into focus, the microscope is then switched to the appropriate filter (green or red, depending on the dye used to end-label the probes) and *Salmonella* cells are scored visually according to their fluorescence (Figures 2 and 3).
3. For cytometric detection of liquid surface miniculture samples, various instruments may be used, depending on local availability. In our lab, PBS-suspended samples are transferred to 5 mL round bottom sampling tubes (BD Falcon) and examined using a FACSCanto flow cytometer with excitation at 647 nm (Materials Table). For enriched samples containing high numbers of total bacteria, the "low flow rate" setting (10 µL min^{-1}) is used and 5,000-50,000 events are collected. Data are analyzed using FlowJo software (version 8.8.6, Tree Star, Inc., Ashland, OR) or other suitable analysis software (Figure 4, panels A and B).

6. Representative Results

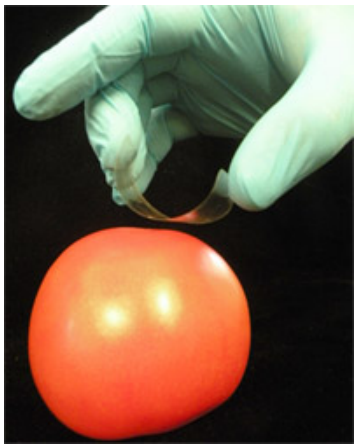

Figure 1. Use of adhesive tape for sampling of *Salmonella* spp. from the surface of an artificially contaminated tomato.

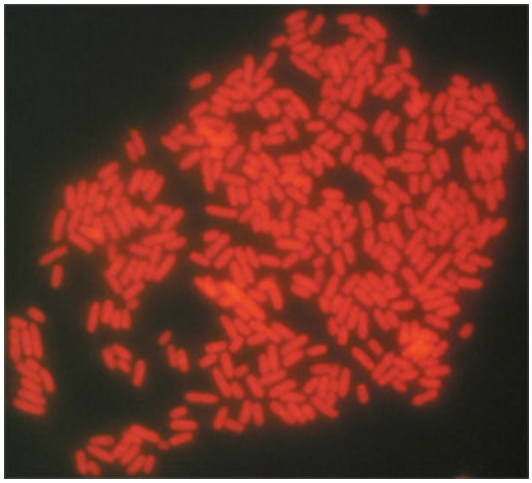

Figure 2. Typical results for direct-to-tape sampling and FISH detection of *Salmonella* Typhimurium ATCC 14028 from the surface of a tomato (100 X oil objective). A two-probe cocktail of Texas Red-labeled probes (Sal3/Salm-63) was used to label these cells.

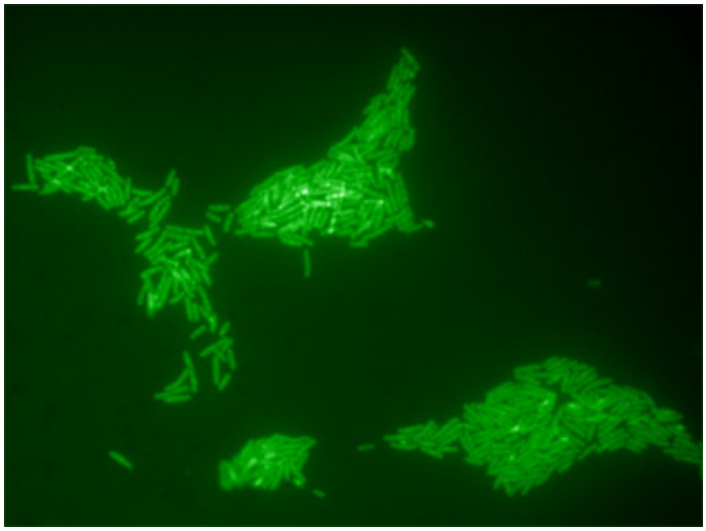

Figure 3. Microcolonies of *Salmonella* Typhimurium ATCC 14028 formed on the surface of Xylose Lysine Tergitol-4 (XLT-4) agar after an 8 h on-tape enrichment at 37°C. The initial inoculum was sampled from the surface of an artificially contaminated tomato. Solid-phase enrichment increases the numbers of cells available for detection and also enhances cellular rRNA content.

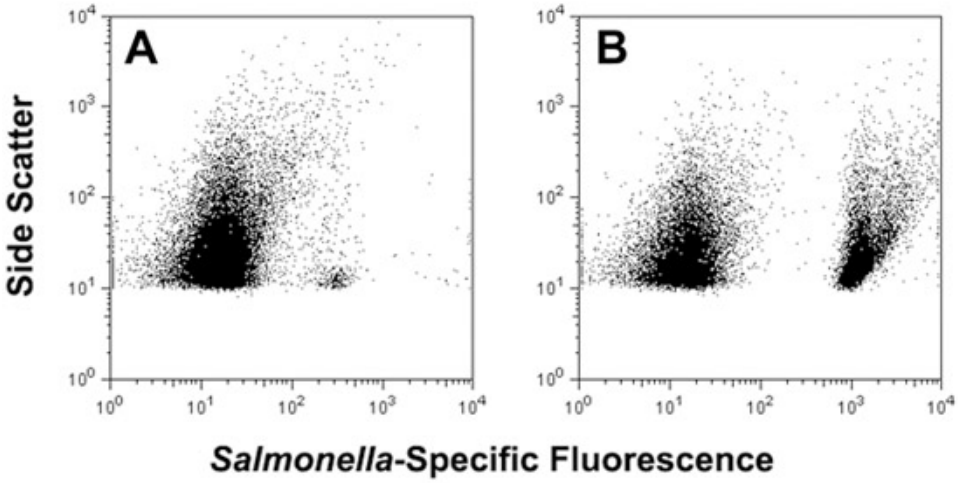

Figure 4. Use of adhesive tape for sampling of *S. enterica* serovar Typhimurium from the surface of an artificially contaminated tomato, followed by direct analysis via FISH and flow cytometry (panel A) or after 5 h non-selective liquid surface miniculture enrichment in a perfusion chamber filled with 500 µL Trypticase Soy Broth (panel B).

Discussion

Simple and rapid methods for detection of pathogens on produce surfaces may help mitigate foodborne disease by providing timely and actionable data. Adhesive tape-based sampling methods have been used in environmental, clinical and food microbiology since the 1950's and involve pressing of "Scotch"-style tape to surfaces for removal of microorganisms, followed by direct microscopic examination or transfer of adherent microorganisms to solid media for growth (Barnetson & Milne, 1973; Edwards & Hartman, 1952; Evancho *et al.*, 2001; Fung *et al.*, 1980; Lakshmanan & Schaffner, 2005; Langvad, 1980). A recent modification described the combination of tape-based sampling with fluorescence *in situ* hybridization (FISH) for culture-independent analysis of microorganisms colonizing the surfaces of stone monuments (La Cono & C. Urzì, 2003). We have applied a similar approach for rapid sampling and detection of *Salmonella* present on fresh produce, including tomatoes, spinach and Jalapeño peppers (Bisha and Brehm-Stecher, 2009a). Adhesive tape can be used to remove cells from these foods and samples can then be processed for FISH and viewed via fluorescence microscopy. In this way, as few as 10^3 CFU cm^{-2} *Salmonella* (the limit of detection for microscopy-based methods) can be detected on fresh produce within ~1.5 - 2 h. Alternatively, short (8 h) solid phase enrichments can be performed by placing the cell-charged tape face down on selective agar, and the resulting microcolonies detected by FISH. By overlaying the tape with ≤ 500 µL liquid media, tape-sampled *Salmonella* cells can be detached and detected directly after FISH using flow cytometry (Figure 4, panel A). Brief incubation (~5 h) of these non-selective liquid minicultures allows substantial enrichment of bacteria, with *Salmonella* cells easily detected in complex mixtures of target and non-target cells after FISH (Figure 4, panel B). Collectively, our results demonstrate that this approach provides a novel method for extraction, presentation and identification of specific bacteria present on tomatoes and other fresh produce. Although we have described the use of DNA-based probes here, it is likely that this approach may also be expanded to include

alternative probe chemistries, such as peptide nucleic acids (PNAs), for which *Salmonella*-specific probes have also been described (Almeida *et al.*, 2010).

Disclosures

No conflicts of interest declared.

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References

1. Almeida, C., Azevedo, N.F., Fernandes, R.M., Keevil, C.W., & Vieira, M.J. Fluorescence *in situ* hybridization method using a peptide nucleic acid probe for the identification of *Salmonella* spp. in a broad spectrum of samples. *Appl. Environ. Microbiol* 76: 4476-4485 (2010).
2. Barnetson, R.S. & Milne, L.J.R. Skin sampling for *Candida* with adhesive tape. *Br. J. Dermatol.* 88, 487-491 (1973).
3. Bisha, B. & Brehm-Stecher, B.F. Simple adhesive-tape-based sampling of tomato surfaces combined with rapid fluorescence *in situ* hybridization for *Salmonella* detection. *Appl. Environ. Microbiol.* 75, 1450-1455 (2009a).
4. Bisha, B. & Brehm-Stecher, B.F. Flow-through imaging cytometry for characterization of *Salmonella* subpopulations in alfalfa sprouts, a microbiologically complex food system. *Biotechnol. J.* 4, 880-887 (2009b).
5. Edwards, R.W., & Hartman, E. A simple technique for collecting fungus specimens from infected surfaces. *Lloydia*. 15, 39 (1952).
6. Evancho, G.M., Sveum, W.H., Moberg, L.J. & Frank, J.F. Microbiological monitoring of the food processing environment. In F. Pouch Downes and K. Ito (eds.) Compendium of Methods for the Microbiological Examination of Foods, 4th edition, American Public Health Association, Washington, D.C. (2001).
7. Fung, D.Y.C., Lee, C.Y. & Kastner, C.L. Adhesive tape method for estimating microbial load on meat surfaces. *J. Food. Prot.* 43, 295-297 (1980).
8. Kutter, S., Hartmann, A., & Schmid, M. Colonization of barley (Hordeum vulgare) with *Salmonella enterica* and Listeria spp. *FEMS Microbiol. Ecol.* 56: 262-271 (2006).
9. La Cono, V., & Urz, C. Fluorescent *in situ* hybridization applied on samples taken with adhesive tape strips. *J. Microbiol. Meth.* 55, 65-71 (2003).
10. Lakshmanan, C., & Schaffner, D.W. Understanding and controlling microbiological contamination of beverage dispensers in university foodservice operations. *Food Prot. Trends* 26, 27-31 (2005).
11. Langvad, F. A simple and rapid method for qualitative and quantitative study of the fungal flora of leaves. *Can. J. Microbiol.* 26, 666-670 (1980).
12. Nordentoft, S., Christensen, H. & Wegener, H.C. Evaluation of a fluorescence-labelled oligonucleotide probe targeting 23S rRNA for *in situ* detection of *Salmonella* serovars in paraffin-embedded tissue sections and their rapid identification in bacterial smears. *J. Clin. Microbiol.* 35, 2642-2648 (1997).

