Three-dimensional visualization of *Salmonella* attachment to poultry skin using confocal scanning laser microscopy

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K.Y. KIM, J.F. FRANK AND S.E. CRAVEN. 1996. The objective of this study was to locate the position of attached or entrapped *Salmonella* cells in poultry skin. Confocal scanning laser microscopy (CSLM) was used to obtain optical sections of intact poultry skin without artefacts associated with dehydration and other sample preparation techniques. A technique was developed to prevent compression of the poultry skin during CSLM operation. Images of bacteria and poultry skin were obtained after staining with Pyronin-Y. Data indicated that *Salmonella* cells were mostly located in the cervices and feather follicles. *Salmonella* in feather follicle floated freely in surrounding liquid even after the skin was thoroughly rinsed.

INTRODUCTION

The presence of *Salmonella* on poultry skin has caused public health concerns. During the processing of poultry, cross contamination occurs (Green 1987), resulting in a significant increase in *Salmonella* incidence on carcasses exiting the immersion chiller (Lillard 1990). Kim *et al.* (1996a,b) suggested that attachment of *Salmonella* to poultry skin is mostly non-specific and physical structure of poultry skin should be the most important factor preventing detachment. This study was undertaken to determine if there are specific sites on poultry skin for *salmonella* attachment. Confocal scanning laser microscopy (CSLM) achieves greater resolution than conventional light microscopy by rejecting out-of-focus-plane scattered light, (White *et al.* 1987). The use of high intensity laser light allows obtaining external and internal 3-D information by optical sectioning of thick specimens (Brakenhoff *et al.* 1988). The electron microscope provides better resolution, but normally requires that the sample be dehydrated, embedded in plastic, or shadowed with a metal coating (Crang 1988). CSLM makes it possible to observe samples in the fully hydrated state without complex sample preparation methods (Blonk and van Aalst 1993).

MATERIALS AND METHODS

Specimen preparation

Skin pieces (2 × 2 cm) were removed from the same location on breasts of freshly plucked broilers (Gold Kist, Athens, GA, USA). *Salmonella typhimurium* (ATCC 14028) was maintained on brain heart infusion (BHI) agar (Difco). Inocula were prepared by growing cells on BHI broth for 20 h at 37°C. The outer surfaces of broiler skin pieces were exposed to the 10⁶ cfu ml⁻¹ cell suspension for 2 h and loosely attached cells were then rinsed off using a 10 ml pipette. An electrical pipetter (Pipet-aid, Drummond Scientific Co., Broomall, PA, USA) was used to provide constant rinsing pressure. After rinsing, skin was stained with 0.01% (w/v) Pyronin-Y (Sigma) for 10 min and then rinsed by flooding with 0.85% (w/v) saline solution for 10 min to remove uncombined stain residues.

A technique was developed to prevent compression of the poultry skin during CSLM operation. A stained skin piece was put on a microscope-slide and then covered with thin plastic plate (1.5 × 1.5 cm; 0.25 mm thick) with a square hole (0.5 × 0.5 cm). One drop of mounting oil was applied through the hole which was then covered with a coverglass avoiding air bubble formation. Then each side of the coverglass was fixed with scotch tape. The specimen was observed through the coverglass on the square hole.

Image acquisition

After fluorescent staining and specimen preparation, the chicken skin was observed using a BioRad MCR-600 CSLM (BioRad, Hemel Hempstead, Herts, UK). A 15 mW argon/crypton ion laser was used as a light source. The excitation wavelength for Pyronin-Y was 568 nm. The microscope was equipped with a 60X Nikon objective lens (PlanApo60; NA = 1.40). Stacks of 2-D optical sections were collected.
and stored. This was done by digitizing a sequential series of images while focusing down through the specimen using a computer controlled stepping motor. Each optical section contained 768 × 512 pixels. To remove visual noise, each optical section was scanned three times and averaged using Kalman algorithm (Jain 1989).

3-D volume reconstruction

A 3-D volume reconstruction technique was used to improve visualization of Salmonella cells entrapped in feather follicles. Data sets of multiple optical sections were transferred to a Silicon Graphics Personal IRIS D/35G computer. Data sets were reconstructed using Voxel View Software (Vital Images, Fairfield, IA, USA). A fast implementation of the volume rendering technique was used (Levoy 1988). Angle, contrast, opacity and threshold of reconstructed image were modified for improved visualization.

RESULTS AND DISCUSSION

Pyronin-Y staining provided the best simultaneous visualization of Salmonella cells and poultry skin among several fluorescent dyes tested including Nile Red, rhodamine, acridine orange, fluorescein isothiocyanate (FITC) and fluorescent antibody (FA-Salmonella poly O). The specimen preparation technique prevented compression of the skin during CSLM data collection. Attachment is generally considered to be a two-step process: reversible association with a surface followed by irreversible adherence. In this study, the skin was rinsed to remove reversibly attached bacteria. After 2 h inoculation with 10⁹ cells ml⁻¹ concentration, most salmonella cells attached on the flat portion of the skin surface were readily rinsed off. Salmonella cells remaining on the poultry skin surface after rinsing were mostly located in crevices (Fig. 1). Optical sectioning of inoculated chicken skin shows Salmonella cells entrapped in the feather follicles even after rinsing (Fig. 2a). Salmonella cells were entrapped deep inside the feather follicle with water (Fig. 2b). Salmonella cells could be observed in any depth of the feather follicles. This observation indicates that even unattached, floating Salmonella cells in the entrapped water cannot be easily washed out. Lillard (1989) reported recovery of bacteria after 40 consecutive rinses of the same carcasses.

To improve visualization of entrapped Salmonella through 3-D volume reconstruction, the stepping motor increment was reduced from 1.0 to 0.3 μm (Fig. 3). The majority of entrapped cells remained unattached to the side of the feather follicle. During the time (approx. 5 min) required to collect the 474 optical sections used for the volume reconstruction, Salmonella cells rotated and moved to a limited extent. This resulted in the cells’ size appearing larger than the actual size (Fig. 3). This observation confirms that entrapped water in the feather folicle can be a significant source of Salmonella during the immersion chilling process of poultry.

Kim et al. (1996a) reported that cell viability and cell

Fig. 1 CSLM micrograph of typical chicken breast skin surface inoculated with Salmonella typhimurium (arrows). The bacterial cells remained mostly in the crevices after rinsing the skin surface. Pixel size = 0.138 μm; scale bar = 10 μm

Fig. 2 CSLM micrographs of a feather folicle on chicken skin inoculated with Salmonella typhimurium (arrows). Pixel size = 0.275 μm; scale bar = 40 μm. (a) The optical plane is 20 μm below the skin surface. (b) The optical plane is 30 μm below the skin surface

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surface factors do not play significant roles in *Salmonella* attachment to poultry skin. This result indicated that physical structure has a more significant role than bacterial surface factors. Thomas and McMeekin (1982) reported that water-immersion cleaning and chilling of poultry carcasses caused the skin to swell by taking up water and exposed deep channels and crevices in the skin surfaces. Although water uptake has been known as a significant source of contamination (Lillard 1986; Thomas and McMeekin 1984) and relations between skin microtopography and *Salmonella* contamination have been investigated by using electron microscopy (Thomas and McMeekin 1981; Kim and Doores 1993), there has been no actual visualization and determination of entrapped water as a source of contamination.

This study demonstrates the potential of CSLM for the study of bacterial contamination of poultry skin. The entrapment of *Salmonella* in feather follicles was observed in fully hydrated, unfixed skin specimens for the first time. Future research should focus on preventing entrapment of contaminated water.

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REFERENCES


