Feasibility of fluorescent detection of pathogens on pork carcasses

Möglichkeiten zum Fluoreszenznachweis von Pathogenen auf Schlachtkörpern von Schweinen

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Summary:
The direct immunofluorescent detection of pathogens on pork skin is evaluated. Calibrate contamination of pork skin with Salmonella Typhimurium (ST) and Listeria monocytogenes (Lm) is developed in 2 h at 4 °C. Then a specific indirect immunofluorescent staining protocol is optimized in order to obtain specific and intensive signals able to be detected by electronic cameras (deported microscopy). Despite the individual staining of ST and Lm is possible on pork skin and is specific and bright, the deported microscopy failed to detect these particles. After respectively 3 and 6 h, we obtain micro-colonies of ST and Lm. Due to the limited power of the video camera used, only the microscope permits the detection on the skin. However, our work gives standard conditions to mime the pathogens contamination and staining directly on a biological matrix such as pork skin. This work is a first step in the development of direct and rapid detection of pathogens on biological matrix.

Keywords: Meat, rapid measure, Salmonella, Listeria monocytogenes, food safety

Introduction
In recent years, outbreaks of bacterial disease due to the consumption of contaminated meat products have increased the demand for new approaches to pathogens control no more only on the products but on the entire production chain from farm to table. Despite of the efforts made for food safety control measures, contamination problems of meat or fresh products by bacteria such as Salmonella spp. or Listeria monocytogenes still remain in many meat productions. Whatever
the control of Salmonella in pork production, a few positives pigs or batches will still be found at the slaughterhouse. At the moment, there is no way to detect pathogens on carcasses in a delay convenient with real time management of food safety assurance system. The serological testing gives an indirect evaluation of the risk. Bacteriology instead of the presence of rapid methods cannot give a positive result within less than 2 days. Under these conditions, the result is available when carcasses are already cut or even eaten. So we aimed to prospect the possibility of staining directly the bacteria on skin, coupled with detection with CCD camera and electronic zooms. We first standardized the conditions to obtain control contaminated coupons of skins with two pathogens as Salmonella spp. or Listeria monocytogenes. Then, we tested specific immunofluorescence techniques which can give specific images of pathogens on the skin. To amplify the intensity of the fluorescent signal, different strategies were tested.

Materials and methods

Strains
Salmonella Typhimurium (ST) strain was obtained from a pig herd and Listeria monocytogenes (Lm) (serotype α2 a) from the environment of a pork-meat cutting plant. These strains were isolated and serotyped at the laboratory of the French Agency for Food Safety. Both strains were kept in deep freeze conditions, regenerated before use 24 h at 30 °C in Brain Heart Infusion (BHI) and subcultured about 18 h in BHI at 30 °C to obtain a 10^7-10^8 cfu/ml suspension.

Pork skins
Pork skins correspond to the surface of commercial dressed carcasses before the first chilling. Scalps of skin were performed on the shoulder and were transported at 4 °C, then frozen at -30 °C except when otherwise specified. Before use, the skin fragments were cut in about 1 cm² pieces and kept 30 min at room temperature in a humidified atmosphere. All the skins used were monitored in order to assess they were free of Salmonella and Listeria monocytogenes in 25 cm² using the technique routinely applied in the laboratory.

Adherence tests
For the adherence tests, the skin coupons were maintained 30 min at 4 °C. Bacterial suspension in Phosphate Buffered Saline (PBS 0,1M, pH 7.2) from 10^6 to 10^8 cfu/ml were used for contamination (10 μl were deposited). After 30 or 120 min incubation, the skin coupons were rinsed 5 times (3 min in 20 ml of PBS) and scraped with a sterile scalpel blade. Scraping material was homogenised in 100 ml of PBS. Bacteria from the rinses and the scraping were enumerated by spiral plate counts on Palcam and Rambach agar for Lm and ST incubated at 37 °C 48 and 24 h, respectively, to evaluate the adherent part of bacterial population.
Immunofluorescence

The skin coupons were fixed overnight at 4 °C in paraformaldehyde (Sigma, 4 %, pH 7.6) then rinsed twice in PBS at room temperature under gentle shaking, then saturated 30 min in a 1 % Bovine Serum Albumine (BSA, Sigma), 0.5 % TX-100® (Sigma) solution in PBS. Successive 40 min incubations were conducted at room temperature under gentle shaking. Rabbit antiserum against the O4-5 somatic antigen of ST (Pharmacia) and rabbit antiserum against the OI/II somatic antigen of Lm (Eurobio) were diluted at time of use 1/30 and 1/50, respectively, for ST and Lm in a 0.1 % solution of BSA and 0.5 % TX-100® in PBS (PBT). Secondary antibodies (anti-rabbit IgG, AlexaFluor 488®) were coupled and used at the recommended dilution in PBT.

Amplification of the signal

Microcolonies

After the 5 rinses realised for the adherence test but without scraping of the surface, the coupons of skin were maintained in different temperature conditions (2, 8, and 30, or 37 °C, respectively, for Lm and ST) and time (from 30 min to 6 h) in a humidified atmosphere.

Fluospheres

Passive adsorption of the O4-5 anti salmonella antiserum on Fluosphere® sulfate microspheres (1 μm, yellow-green fluorescent, Molecular Probes) was validated by selective agglutination in presence of ST. The coated Fluospheres® were used at varying concentrations in PBT.

Observation

The coupons after a last PBS rinse were drained, covered with a drop of immersion oil and observed with an epifluorescent illumination using a microscope (Olympus BH-2) fitted with X40 objective lenses. For depered microscopy, the computer vision system was composed of a Sony DXC LS1P camera with a 1/4" CCD sensor and a motorised zoom OPTEM 100D. The acquisition was realised by a Matrox frame grabber Genesis LC and a PC Computer. The light was emitted by a cold light source Schott KL 2500 LCD fitted with an optic fibber and a low-pass filter (-50 % over 500 nm wave length). The whole system was enclosed in a black box to avoid any interfering light. The sample was placed on a device with a micrometric adjustment.

Results

Adhesion

We chose to calibrate contamination of the skin with two pathogens of interest in public health. On coupons of skin of 1 cm² we dropped a suspension of ST or Lm.
The number of bacteria in the suspension was stable at 4 °C for more than the 120 min of the experiment. We tested a range of *Salmonella* number varying from $7 \times 10^5$ to $7 \times 10^7$ CFU in 10 µl. As shown in Table 1, after 30 min the proportion of ST present in the rinses was about 50% of the deposit and the number of cell on the coupon was very low: always below 5% of the deposit. These proportions did not vary between the inoculation rates. After 2 h if the proportion of rinses cells was again about 50% for the higher deposit, it fell at 35 and 40% respectively for $7 \times 10^4$ and $7 \times 10^3$ ST. The population count in the surface scraping was higher after 2 h than after 30 min for all the deposits. Comparing the evolution of cell counting in the successive rinses and surface scraping revealed differences between ST and Lm (Figure 1). For ST, the two first rinses were efficient and the three following ones were quite stable. The scraping contained about 10 times more ST than the fifth rinse. For Lm, each rinse eliminated regularly bacteria from the surface of the skin. When scraping the surface, the number of Lm was about the same as the number of bacteria present in the last rinse.

**Detection protocol**

The detection protocol allowed us to detect ST or Lm directly on pork skin. The specificity of the signal was clearly shown by the lack of detection when replacing the specific antiserum by another from the same host but directed against another serogroup (we used anti-O9 and anti-OIX as controls for ST and Lm). Optimising the conditions of staining lead to obtain the more intense signal background ratio in less than 4 h. Aiming to amplify the signal we chose two strategies. The use of fluospheres did not allow to detect the bacteria present artificially on the skin, their repartition is not specific all over the surface of the skin. At last we tried to obtain a physical amplification of the signal. Bacterial growth created micro-colonies that amplified the final signal. On the early minutes of incubation, the bacterial population on the skin was a succession of individual cells. This was still the case after 8 h incubation at 2 °C. Micro-colony formation of Lm started after 2 h and structures of about 30 bacteria were obtained after 4 h at 30 °C (Figure 2). Comparing this course, ST formed micro-colonies of 20 bacteria in 2 h at 37 °C (Figure 3a) and after 4 h of incubation at 37 °C (Figure 3b), the population lost the organization of individualized micro-colony structures to turn on a biofilm formation. The more rapid micro-colony formation corresponded to 37 °C for ST and 30 °C for Lm.

We obtained very intense fluorescent spots associated with ST or Lm on pork skin within 4 h. We tried to detect these signals with the deported microscopy. Automatically analysed images of specifically coloured bacteria on skin was difficult because the pig skin itself emitted a light causing interference. This noise did not allow us to obtain sufficiently contrasted images as shown with the spheres (Figure 4). Thus an automatic analysis was not possible.
Discussion

The presence of pathogenic bacteria on the skin may lead to cross contamination all along the production chain. A commonly used strategy is to detect the positive pigs at the herd level. This strategy was supported by an increasing number of states as a function of the seropositivity of pigs quantified at the herd. But at the slaughterhouse, the contamination is a problem of quantity: if the contamination is about 0.1 *Salmonella* per cm² for more than 75% of contaminated carcasses (Oosterom et al., 1985), it is already a problem for the industrials. So an alternative is to detect as soon as possible in the process of carcasses dressing, the batches giving the 25% of more contaminated carcasses. Detection of bacteria with a CCD camera has already been exposed. Yasui and Yoda (1997) detected single bacteria after filtration of a pure culture on a membrane. We detected fluorescent particles of about 1 μm in diameter on skin coupon. So we tried to connect the specificity of the antibody-antigen association with the brilliant intensity of these micro-spheres. At the moment, it appears that despite the association of the antibodies with the sphere, as being demonstrated by agglutination in presence of bacteria, we could not realize the association of the sphere with *Salmonella* on the skin. As we needed an amplification of the signal associated with *Salmonella* or *Listeria*, we tried to obtain micro-colonies directly on the skin. As previously described by Rodrigues and Kroll (1990) as well as by Yasui and Yoda (1997), the micro-colony immunoluminescence method increased dramatically the signal to noise ratio. So we developed this strategy aiming to reach the sensibility threshold of the deported microscopy.

Attachment of *Salmonella* on skin was more studied in poultry than in pork. In 1986, Lillard described the transfer of bacteria from the liquid part to the skin due to water uptake when immersing the carcasses. The phenomenon appeared to be passive (Kim et al., 1996) and the maximum proportion of "attached" bacteria reached 61% of the number of deposited bacteria after 60 min of incubation. In pork processing, the adherence of *Salmonella* on skin from immersion water is not of concern. But using spray chilling, the water film on the surface could mimic the immersion and facilitate cross contamination. Our work allowed us to obtain, for the first time to our knowledge, a standardized artificial contamination of pork skin by *Salmonella* and *Listeria monocytogenes*. It appears that adherence was obtained on pork skin in about the same conditions (about 40% of adhering bacteria after 2 h of contact) despite the differences in micro-topography of those two kinds of skins.

The ways of micro-colony formation on pork skin differ for *Listeria* and *Salmonella*. The optimal incubation time was about 4 h for *Salmonella*. After this
delay, we observed a lose of structural organization, getting from micro-colonies to a biofilm structure. This reorganization was already demonstrated after 8 h incubation as a consequence of activity of type IV pili for other bacteria (Knutton, 1999). The type of pilus is common in the enteropathogenic *Escherichia coli* (Nagayama, 1996) and is expressed for several *Salmonella* serotypes (Sohel et al., 1993). It appeared that the *Salmonella* Typhimurium we used in this study exhibited this phenomenon on skin after artificial contamination. The ability of *Listeria monocytogenes* to colonise many surfaces of agro-industrial concern (Somers et al., 1994), including meat products (Takeuchi et al., 2000) is well known. As shown in this study, *Listeria monocytogenes* attachment appears very reversible, the delay to obtain micro-colony was about 8 h and we were not able to obtain biofilm reorganization. The micro-colony formation from single bacterial cells on skins was, as shown in our work, function of time, temperature and species.

The plasticity of the immunodetection technique after micro-colonisation or on single cells allowed to obtain different levels of detection. For example, Rodrigues and Kroll (1990) chose the use of a panvalent ‘Bacto FA’ antiserum able to detect all *Salmonella*. Hoszowsky et al. (1996) used pools of monoclonal antibodies to LPS core epitopes recognizing serogroups B, C, D and E of *Salmonella*. These authors gave different levels of detection from a unique matrix: poultry carcasses rinses. Our purpose was to detect on pork skin a *Salmonella* Typhimurium. We chose a somatic O4,5 antiserum detecting all bacteria of serogroup B and only these ones. The specificity limit of the technique depends only on the specificity of the used antibodies. Actually we are able to detect separately *Salmonella* and *Listeria monocytogenes* on skin after artificial contamination. As these bacteria are not closely related we are not exposed to cross reactivity with the used serum. As a consequence we’ll try to obtain a simultaneous detection of both pathogens on different supports, naturally contaminated, only on the basis of emission wave length of the fluorochromes used. As we obtained an intense and specific staining of micro-colonies, we thought of using deported microscopy for detection directly on skin. At the moment the material we own is not enough sensitive to catch the signal on the skin. Using a monochromatic light source with a specified wavelength corresponding to the excitation wavelength of the used dye (like a laser light) could allow to reduce the part of the light emitted by the pig skin. But, the cost of such a device would not be competitive considering the existing solutions. In conclusion, direct immunofluorescent staining on pork skin is possible, rapid and the simultaneous detection of different species (realized within 30 h by combination of culture and PCR (Peng and Shelef, 2001) will be possible by immunofluorescence within 4 h. Actually this detection is observed with a microscope, waiting for development in electronic camera and zooms sensitivity.
Acknowledgements

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References


Table 1
Effect of contact time at 4 °C on the adhesion of different deposits of ST on pork skin coupons

<table>
<thead>
<tr>
<th>Contact time</th>
<th>Deposit in 10 μl</th>
<th>70,70</th>
<th>%</th>
<th>7,07</th>
<th>%</th>
<th>0,71</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>30 min</td>
<td>Mean rinses</td>
<td>35,30</td>
<td>± 6,2</td>
<td>49,93</td>
<td>4,00</td>
<td>± 0,78</td>
<td>56,58</td>
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<tr>
<td></td>
<td>Mean scraping</td>
<td>2,60</td>
<td>± 1,3</td>
<td>3,68</td>
<td>0,18</td>
<td>± 0,13</td>
<td>2,55</td>
</tr>
<tr>
<td>120 min</td>
<td>Mean rinses</td>
<td>35,70</td>
<td>± 7,8</td>
<td>50,50</td>
<td>2,45</td>
<td>± 0,22</td>
<td>34,65</td>
</tr>
<tr>
<td></td>
<td>Mean scraping</td>
<td>15,80</td>
<td>± 6,1</td>
<td>22,35</td>
<td>1,80</td>
<td>± 0,44</td>
<td>25,46</td>
</tr>
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</table>

Means (n=3) and ± s.d. (standard deviation) in *10^4 CFU are depicted.

Figure 1
Evolution of bacterial population in successive rinses and after scraping
Figure 2
Listeria monocytogenes microcolonies on pork skin after 4 h at 30 °C; a), microcolony, b), in another focal plane, c), individual Lm non initiating microcolony formation, and d), bacteria of the skin not stained (bar is 10 μm).

Figure 3
Micro-colony of about 30 Salmonella Typhimurium obtained on pork skin after artificial contamination, after incubation a), 2 h, and b), 8 h at 37 °C, and stained with the optimized indirect immunofluorescent protocol (bar is 10 μm).
Figure 4
Light intensity needs of the deported microscopy as shown by comparison of the 1 μm in diameter Fluospheres observed on pork skin with a), the microscope, and with b), the electronic zoom and CCD camera (bar is 10 μm).