ISOLATION AND CHARACTERISATION OF LISTERIA MONOCYTOGENES FROM PIGGERIES IN FRANCE.

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Abstract
Recent analyses of human listeriosis outbreaks showed that these were associated with consumption of delicatessen items or pork products from other sources. Serotyping of implicated strains demonstrated that they belonged mainly to serotype 4b. Serotype 4b Listeria monocytogenes was rarely among isolates from slaughterhouses, which belonged mainly to serotype 1/2a. A study was carried out on 32 pig herds to describe the situation regarding this pathogen. Combining different sampling sites Listeria monocytogenes was found in 6 (18.7%) of the herds. Considerable strain diversity among isolates from the 6 herds (6 different serotypes) was showed. Moreover a herd carrying strains of serotype 4b was identified. Using RFLP-PFGE we were able to describe a difference between strains of this serotype. The study suggests that the role of pigs as a primary source of serotype 4b strains in the pork production chain should be reconsidered.

Introduction
Foodborne diseases are a significant public health concern in many countries. Whether or not is listeriosis associated more with meningitis, neonatal mortality and abortion than gastrointestinal disturbance, Listeria monocytogenes is well recognised as a cause of foodborne outbreaks. The main food sources have been known for many years [1, 2] and involve the consumption of cheese and vegetables [3], but epidemiological investigation of recent outbreaks clearly associated cases with consumption of delicatessen items and further processed pork products from other sources [4-6]. Nowadays measures taken to control potential pathogens in the food production chain tend to follow a “stable-to-table” approach. Many studies have characterised the strains of Listeria isolated at different stages of the pork production chain including cutting plants and slaughterhouses [7, 8]. It appears that a major clone of Listeria monocytogenes is well adapted to pork production, and perhaps to meat in general since it could also be found in poultry processing plants [9]. This related population belongs to serotype 1/2a and represent a major clone that is not involved in human listeriosis, where serotype 4b is more often the causative agent, particularly in recent French outbreaks [4-6]. A few strains that differ from the 1/2a major type can be traced from cutting plants to slaughterhouse [7-8]. Since product contamination may originate in the slaughterhouse the potential role of live pigs as the ultimate source of Listeria monocytogenes need to be considered. Recent reports on Listeria monocytogenes in slaughterhouses made no mention of serotype 4b [8, 9]. Because the prevalence at herd level is thought to be low, it appears difficult to trace back contamination with Listeria monocytogenes to the farm. The aim of this study was to investigate serotype variability among Listeria monocytogenes strains isolated from pig herds and to compare the isolates with some strains associated with human infections.

Materials and Methods
Isolation of Listeria monocytogenes
Thirty two different pig herds of market age were investigated for the presence of Listeria monocytogenes by sampling the animals and their rearing environment as follows: the backs of 5 randomly chosen pigs were swabbed, auger and wall surfaces were swabbed independently and 25 g of feed were collected outside the room at the silo or in the mixing machine depending on the method of feed distribution. Detection of Listeria monocytogenes involved the use of half strength Frazer broth as the first enrichment step. The feed sample was diluted 1/10 before examination and, for swabs, 150 mL of the broth were added to the bags. After incubating at 30°C for 24 h, 100µL of the first enrichment were transferred to the full strength Frazer broth (9 mL) and incubated at 37°C for 48h. A loopful of enrichment culture was streaked on Aloa plate and further incubated at 37°C for 24h. Typical colonies on the Aloa plate (maximum 10) were picked, biochemically confirmed as Listeria monocytogenes and stored at -60°C in Tryptic Soy Broth supplemented with 15% glycerol for further characterisation. All media were obtained from AES (France).

Serotyping
In total, 39 isolates of Listeria monocytogenes were obtained from the positive samples. Serotyping was carried out according to the manufacturer’s instructions using O and H antisera Eurobio (France).

Pulse field gel electrophoresis
The DNA fragments were separated on a 1% Seakem agarose gel
in 0.5x solution of Tris-Borate-EDTA (Invitrogen, UK) at 14°C in a CHEF-Mapper PFGE apparatus (Bio-Rad, Hercules, CA). The electrophoretic parameters used were as follows: initial switch time, 4.0s; final switch time, 40.0s; run time, 22h; angle 120°; gradient, 6.0V/cm. After electrophoresis, the gel was stained in 0.5µg/ml ethidium bromide. The resulting DNA patterns were examined on a short-wave UV transilluminator and then photographed for visual analysis. Lambda DNA ladder (New England Biolabs, Beverly, MA) was used as molecular weight marker. Five 4b serotype *Listeria monocytogenes* strains from our laboratory collection were included as standards: CIP 27 793, 4999, 3277 and H544, the two last strains have been associated in previous human listeriosis outbreaks.

**Results** *Listeria monocytogenes* was detected in six of the herds (18.7%). For farm to farm (identified from A to F) the distribution of positive samples varied (Table 1). For two of the herds (B and D) only one site yielded *Listeria monocytogenes*, and this was the feed in each case. For herd F, all the sites sampled were positive for *Listeria monocytogenes* detection. The two most frequently positive sites were the backs of the pigs and the feed. The most frequently isolated strains in these 6 herds belonged to the serotype 1/2a and this was found in all six cases. At farm A, the strains isolated from the animals and in the feed trough were not the same implying a possibly different origin for the strains in this herd. A similar situation was evident in herd E but differing in detail. Here, 3 of the 4 sampling sites were positive. The feed yielded *Listeria monocytogenes* (in 25g) but not the trough swab. From swabs of the pen walls and backs of the pigs, 4 of 6 isolates examined were of different serotypes. At this particular farm serotype 4b was isolated.

A molecular typing method was used to better characterise the serotype 4b field strains and compare them with strains isolated during grouped cases episodes (Fig 1). The strains Eb1, Eb2 and Eb3 were isolated from the animals, strain Ea and Ec came respectively from the feed (sampled in the mixing machine) and pen-walls of the same herd. Since serotype 4b is commonly found in outbreaks of human listeriosis, the profiles of the herd strains were compared with other 4b serotype strains isolated in slaughter plants and others associated with a previous outbreak using macrorestriction analysis followed by PFGE. Using this technique, differences in pattern profile could be observed the profiles. After Apal digestion of the genomic DNA, the restriction pattern obtained for the Ea strain is different from those of the Eb1, Eb2, Eb3 and Ec strains. Upon the 19 bands observed for the Eb1, Eb2, Eb3 and Ec strains and the 16 for the Ea strain, 12 are common and are present in the pattern of the standards. One additional common band is present for CIP27793, 4999, 3277, H544 and Ea strain but absent for the other 4b strains from the herd E. After Ascl restriction analysis, on the 10 bands of its pattern, Ea shares respectively 6 and 8 bands with the other strains of the herd E and the standard strains. The strain isolated from feed presents a different restriction pattern compared to that observed for the animal and environment strains. Ea appears closer to the 4b reference strains than the isolates from animal and pen walls. It could be concluded that the two strain types were of different origins, a conclusion that would not be possible with phenotyping methods.

**Discussion** In this study, two types of samples in association, animals back and the feed, indicated the contamination status of the 6 herds. However to better describe the heterogeneity of contamination (particularly in two of the herds), extending the number of sites sampled would be needed. One of the 6 herds presenting *Listeria monocytogenes* allowed the isolation of 4b serotype strains. This serotype appears to be rare in pork slaughter/cutting plants [9] yet it is very often associated with human outbreaks [4]. Therefore we aimed to obtain characterised the 4b serotype strains more fully.

Nowadays it is recognised that biochemical characterisation and serotyping have reached their limits for discriminating between strains from down-stream sources of the production chain and further better understanding will only be made at the molecular level. So far few attempts have been made to investigate the herd origin of slaughterhouses isolates [10] and to report molecular subtyping of *Listeria monocytogenes* strains from pig herds. Strains isolated from slaughterhouses or cutting plants could be endemic [8, 9, 11], due to surfaces colonising of the organisms [12]. Thus control measures against *Listeria monocytogenes* have been based on the implementation of

<table>
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<tr>
<th>Farm identification</th>
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<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
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<tr>
<td>In 25g of food</td>
<td>nd</td>
<td>1/2a</td>
<td>nd</td>
<td>1/2a</td>
<td>NT+4b</td>
<td>1/2a</td>
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<td>Back of 5 pigs</td>
<td>1/2a</td>
<td>nd</td>
<td>1/2a</td>
<td>nd</td>
<td>1/2a+4b</td>
<td>1/2a</td>
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<tr>
<td>Walls</td>
<td>nd</td>
<td>nd</td>
<td>1/2a</td>
<td>nd</td>
<td>1/2c+4b+4c</td>
<td>1/2a</td>
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<td>Trough</td>
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Table 1 Distribution of positive samples from contaminated farms NT: non-typable strain of *L. monocytogenes*; nd: none detected.
appropriate cleaning and disinfection protocol [11]. One hypothesis suggests that some strains could persist on carcasses throughout the process of pork slaughtering without any need to colonise plant surfaces. However, their presence may be concealed by strains that are present in greater numbers on plant surfaces. Serotype 4b seemed to be less efficient in colonising surfaces than other serotypes [13], and it appears difficult to find this serotype in slaughterhouses [8, 9].

According to our study, the ultimate source of the isolates could be the herd itself. In view of the diversity in Listeria monocytogenes contamination in this study (2/6 herds with at least 2 serotypes and the presence of different 4b serotype strains in one of them), the exact role of pig herds as sources of the organism should be further investigated in relation to public health considerations.

References

Figure 2: PFGE separation of Apa I (lanes 2-5 and 7-11 and Ascl I (lanes 13-16 and 18-22) macrorestriction fragments of genomic Listeria monocytogenes DNA from herd E and laboratory collection. Lanes 2 and 13: CIP 27793, lanes 3 and 14: 4999, lanes 4 and 5: 3277, lanes 5 and 16: H544, lanes 7 and 18: EA, lanes 8 and 19: Eb1, lanes 9 and 20: Eb2, lanes 10 and 21: EB3, lanes 11 and 22: Ec. Lanes 1, 6, 12, 17, and 23: Lambda DNA used as molecular weight marker (Biolabs, New England).