Reactivities towards enterobacterial LPS in pig sera from herds in a Salmonella non-endemic region

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Introduction

In Sweden legislation concerning extensive restrictions in herds found Salmonella positive has been in operation for four decades. All findings of Salmonella isolates among animals are compulsory notifiable and reports of Salmonella findings have been published continuously, first time 1957 (1) and last time 1998 (2). Bacteriological prevalence in herds, slaughter-houses and cutting plants has been documented during the last four years and the overall prevalence has been proven to be less than 0.1%, 95% confidence interval, (2-5). In addition to the national programme Sweden has as well participated in the European project SALINPORK investigating the prevalence and developing strategies to control and reduce Salmonella (6). In this connection sera (3050 in total) from 61 Swedish pig herds were tested in the Danish surveillance system which is based on serological testing rather than bacteriology as in Sweden. A significant part of these sera had moderate responses (20-70 OD%). When applying 10 OD% as cut-off value, 59% of the herds had more than one sero-reactor, and at 40 OD% as cut-off value 7% of the herds had more than one sero-reactor. The mean OD% for all individual pigs in the 61 herds was 4.4, with the standard deviation of 6.7.

In order to investigate the background for these reactivities, immunochemical analyses were performed comparing a selection of these sera from the 61 herds with sera originating from Danish pig herds with verified Salmonella infections.

Materials and Methods

Samples. All sera originated from slaughter pigs (90-100 kg). 61 samples were selected from 13 of the 61 Swedish pig herds, which participated in the serological examination described above. The selected herds all had samples in the range of 20-70 OD%. As references, 35 sera from 3 Danish pig herds with verified Salmonella infections were selected (all samples had OD%>20). For determination of IgM/IgG OD-ratio's only samples with OD>0.3 for either IgM or IgG were included in the calculations.

ELISA procedure. Microtiter plates were coated with Citrobacter freundii LPS or Yersinia enterocolitica O:3 LPS or a mixture of S. Typhimurium LPS and S. Choleraesuis LPS in 0.1 M sodium carbonate, 1.0 M NaCl, pH 9.6 overnight at 4°C. The indirect ELISAs were then performed according to the procedure described in (7). Serum samples were diluted 1:400 in PBS, 0.05% Tween 20, 1% BSA. Duplicates of 100 µL of each serum were applied and incubated for 1 h at room temperature without agitation. The plates were then washed three times in PBS, 0.05% Tween 20 and subsequently incubated with peroxidase-conjugated Rabbit anti Swine IgG (P164, DAKO, Denmark) diluted 1:2000 or peroxidase-conjugated Goat anti-Pig IgM (Bethyl, Texas USA) diluted 1:6000, or peroxidase-conjugated Goat anti-Swine IgG (Kirkgaard & Perry, Maryland USA), diluted 1:2500 for 1 h at room temperature. The plates were then washed as before, and 100 µL substrate (0.01% H₂O₂, 0.66 mg/mL 1,2-O-phenylene diamine dihydrochloride in 0.1 M citrate, pH 5) was added to each well and incubated 10-15 min. The reaction was stopped with 100 µL 0.5 M H₂SO₄ and the optical density was read at 490 nm, subtracting 650 nm (background correction).

Transformation of OD% to calibrated OD-values.

To compare previously obtained mix-ELISA results with ELISA results obtained in this work the following backwards calculation was done:

Calibrated OD-value = \( \frac{\text{OD}\% \times (\text{OD}100\% - \text{OD}\%)}{100} \)
Results

Swedish serum samples and Danish reference sera with OD-values over 20 OD% were tested in indirect ELISA with Citrobacter freundii LPS or Yersinia enterocolitica LPS as coating antigens.

A.

Swedish sera

Figure 2. Comparisons of the reactivities obtained in the mix-ELISA with the IgM/IgG OD-ratio’s determined in the mix-ELISA system. ❍ Swedish sera, ■ Reference sera.

B.
Figure 1. Comparisons of the reactivities obtained in the mix-ELISA with the reactivities towards Citrobacter freundii LPS and Yersinia enterocolitica LPS. A) Swedish sera in Citrobacter ELISA, B) Reference sera in Citrobacter ELISA, C) Swedish sera in Yersinia ELISA, D) Reference sera in Yersinia ELISA.
The Swedish sera reacted stronger towards the Citrobacter freundii LPS than the reference sera. The reactivities towards Yersinia enterocolitica LPS were also higher on average for the Swedish sera than for the reference sera. In Fig. 1 the reactivities of the Swedish sera towards Citrobacter freundii and Yersinia enterocolitica are compared with the reactivities obtained in the mix-ELISA (done previously). In the case of the Swedish sera, only a weak correlation existed between the mix-ELISA OD’s and the Citrobacter ELISA OD’s (r=0.30, n=61; p<0.01). In the Yersinia ELISA a very broad range of reactivities was seen for all levels of response in the mix-ELISA for both groups of sera (r= -0.21).

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<tr>
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<th>Swedish sera</th>
<th>Reference Sera</th>
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<tbody>
<tr>
<td><strong>Average IgM/IgG</strong></td>
<td>3.0 (0.4-18.5)</td>
<td>0.4 (0.2-1.5)</td>
</tr>
<tr>
<td>% samples with IgM/IgG &lt;1</td>
<td>20 (10/49)</td>
<td>94 (33/35)</td>
</tr>
<tr>
<td>n</td>
<td>49</td>
<td>35</td>
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**Table 1. IgM/IgG OD-ratio’s obtained in the mix-ELISA.**

![Figure 2. Comparisons of the reactivities obtained in the mix-ELISA with the IgM/IgG OD-ratio’s determined in the mix-ELISA system. ? Swedish sera, I Reference sera.](image)

The IgM/IgG OD-ratio’s were compared to the responses previously obtained in the mix-ELISA (see Fig. 2). For the Swedish sera high IgM/IgG OD-ratio’s were related to low responses in the mix-ELISA. In the reference group of sera, which had more varied levels of response in the mix-ELISA, only a few samples with IgM/IgG > 1 were found.

Relating the calibrated OD-values in the mix-ELISA with the separate OD-values in the IgM and IgG ELISA. The following slopes were obtained:

- $a_{(IgG)} = 0.35$ and $a_{(IgM)} = -0.17$ for the Swedish sera and $a_{(IgG)} = 0.63$ and $a_{(IgM)} = 0.08$ for the reference sera.

**Discussion**

A selection of Swedish sera, which has been found reactive in the Danish mix-ELISA, was examined for reactivities towards other enterobacterial LPS antigens. By indirect ELISA it was found that the Swedish sera had a more varied repertoire of serum antibodies than the reference sera from Danish Salmonella infected herds. Since Citrobacter freundii shares a structural motive in the O-antigenic part of LPS with S. Typhimurium, a cross-reaction of Citrobacter specific antibodies could be expected in the mix-ELISA, but our results showed that high responses in the Citrobacter ELISA were only weakly related to the levels of reactivities in the mix-ELISA. So the relatively higher level of Citrobacter reactive antibodies in the Swedish sera do not explain the observed reactivities in the mix-ELISA. The reactivities obtained in the Yersinia enterocolitica ELISA were independent of the reactivities in the mix-ELISA, since the correlation coefficient (r) was close to zero.

Furthermore, it was found that the Swedish sera had relatively higher IgM/IgG ratio’s than the reference sera, meaning that immunoglobulin class-shift took place in significantly fewer of these pigs. This could be explained by transient infections leaving only a short period of exposure...
to the antigen. If the immuno-stimulating bacteria originate from feed, they might be inactivated by the processing of the feed and thereby lose the ability to stimulate certain cell populations (e.g. $T_H$ lymphocytes) of the immune system. Previously we have observed that the same Swedish sera had lower avidity indices (functional affinity) than the Danish reference sera (8), which supports the assumption of transient infections or stimulation by non-viable bacteria. Since no guarantied Salmonella free protein is available on the world market, Salmonella antigens or fragments of antigens from different serotypes will be present in the protein feed, but in quantities which are not known. Salmonella is often present in raw feed material. In the last published report 10% of consignments with protein feed of animal origin meant for import to Sweden were culture positive (9), but on the other hand, an efficient control of the feed resulted in very few Salmonella outbreaks with feed born background (9-11).

Relating the IgM responses and the responses in the mix-ELISA, also showed that sera with high IgM/IgG OD-ratio’s were detected less efficiently than those sera with high IgG content. Since most of the Swedish sera had high IgM/IgG OD-ratio’s and low avidity indices, they may be detected less readily, because the mix-ELISA favours detection of samples with high avidities and probably also with high IgG content.

The discrepancy between the serological results obtained in the Danish mix-ELISA and the bacteriological results obtained in the Swedish surveillance system can be due to the different management systems used in the two countries. One major point of difference is that Swedish pigs have not been subjected to antimicrobial growth promoters for an extensive period of time in contrast to Danish pigs. From 1986 antimicrobial treatments have only been allowed in cases of clinical disease. Since several antimicrobial growth promoters have been used in the same dosage interval as for clinical use in swine (12), a substantial impact on the intestinal microflora can be expected. The microflora in the gut is characterised by a very complex ecology and the antigenic formula is only known for a very limited number of micro-organisms, mainly pathogenic bacteria easy to culture. Exclusive antigenic specificity has never been proven against Salmonella lipopolysaccharides in this respect. So even though we have not been able to discover any strong cross-reactivity towards other Gram-negative bacteria, some other non-Salmonellae organisms could have elicited these Typhimurium reactive antibodies. Applying serology as the only tool for surveillance of low-prevalent regions like Sweden (where prevalence is $<0.1 \%$, (2-5)) would require a very high specificity, and this will probably never be obtained in a test based on LPS, because of epitopic similarities between different Enterobacteria. This work as well as other studies within the European collaboration SALINPORK, investigating the prevalence of Salmonella, taught us that serological tests should be implemented only with extensive caution in areas with low prevalence.

References


