DEVELOPMENT OF AN ELISA FOR THE DETECTION OF SALMONELLA ENTERITIDIS BASED ON A RECOMBINANT DNA FLAGELLAR ANTIGEN

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SUMMARY

A previous study showed that due to cross-reactivity with other Salmonella species, purified Salmonella enteritidis lipopolysaccharide (LPS) was not suitable as a specific capture-antigen in an ELISA for the detection of S. enteritidis antibodies in chicken sera. Since LPS did not give the desired specificity it was examined if flagellar antigens could be used to generate an S. enteritidis specific ELISA. Purified flagella were used to screen chicken sera and the obtained results were compared with those of our LPS-ELISA. Although cross-reactivity with related flagellar antigens was observed, both the sensitivity and the specificity of this flagella-ELISA was very promising. In order to increase the specificity of the flagellar antigen recombinant DNA techniques were used to identify and subsequently produce large amounts of an S. enteritidis specific fragment of the flagellin protein. The results obtained with this flagellin-fragment demonstrate that flagellar epitopes can be used to develop an ELISA that will specifically detect S. enteritidis antibodies in sera and eggs from infected chickens.

INTRODUCTION

Our previous studies with S. enteritidis lipopolysaccharide (LPS) ELISA showed a significant cross-reactivity of the LPS antigen with antibodies against other Salmonella species (1). This cross-reactivity was due to the O:12-antigen, which is a common determinant of serogroups B and D1 of Salmonella. In an attempt to eliminate this cross-reactivity, LPS was isolated from members of the D2-group, who share the O:9 but lack the O:12-antigen. These attempts failed because no detectable antibodies against O:9 were present in S. enteritidis infected chickens; apparently the O:9 antigen is not a dominant antigen.

A second obvious antigen for the development of an S. enteritidis specific ELISA is the flagellar H-antigen. Since, compared to the O-antigens, there is more diversity in the number of possible H-antigens, an ELISA based on this H-antigen might have a higher specificity. As with most bacteria, the flagella of Salmonella is a polymer of a single protein. This protein, called flagellin, contains three domains: two conserved ends which are separated by a highly variable middle part. These conserved ends function in the polymerization of the flagellin into the flagella, whereas the variable middle part contains the H-type specific epitopes (2). Purified flagella may not be suitable to generate an S. enteritidis specific ELISA because of the presence of the conserved regions. Only a fragment containing the serotype specific region should be used as antigen in such an ELISA. This paper describes the identification of the serotype specific region of the flagellin and the subsequent use of this fragment in an S. enteritidis specific ELISA.

MATERIALS AND METHODS

Bacterial strains.

The following strains were obtained from either the National Institute of Public Health and

Environmental Hygiene, Bilthoven, The Netherlands, or the Institute of Animal Health, Boxtel, The Netherlands: S. agona (O:1,4,[5],12; H:f,g,s:[1,2]), S. bergen (O:47; H:i:e,n,z_{15}), S. derby (O:1,4,[5],12; H:f,g:[1,2]), S. dublin (O:1,9,12,[Vi]; H:f,g:-), S. enteritidis (O:1,9,12; H:[f],g,m,[p]:[1,7]), S. gallinarum (O:1,9,12; H:-,-), S. godesberg (O:30; H:g,m:-), S. heidelberg (O:1,4,[5],12; H:r:1,2), S. infantis (O:6,7,14; H:r:1,5), S. monschaui (O:35; H:m,t:-), S. panama (O:1,9,12; H:l,v:1,5), S. typhimurium (O:1,4,[5],12; H:i:1,2), S. virchow (O:6,7; H:r:1,2).

Antisera and monoclonal antibodies

Sera of random selected chickens from commercial flocks with unknown Salmonella status, and from experimentally (per os) infected chickens were kindly provided by J.C. van den Wijngaard and H.M.J.F. van der Heyden (Institute of Animal Health, Boxtel, The Netherlands). Salmonella strains used for the experimental infections were: S. enteritidis, S. typhimurium, S. panama, S. heidelberg, S. agona, S. infantis and S. virchow.

Chicken sera from three known S. enteritidis-positive flocks were kindly provided by W. Braunius (Institute of Animal Health, Velp, The Netherlands).

H-type specific polyclonal antisera (anti-H:g,m and anti-H:m,t) were obtained from Wellcome Diagnostics (Dartford England). Polyclonal H-type specific antisera were a gift of W. Janssen, the National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands. Monoclonal antibodies against the S. enteritidis flagellin (4) were kindly donated by F. Zijderveld, ID-DLO, Lelystad, The Netherlands.

DNA manipulations

All DNA manipulations were performed according to Maniatis et al. (5). The flagellin gene of S. enteritidis was amplified by the Polymerase Chain Reaction (PCR) using Taq DNA polymerase (Promega, Madison, WI). PCR conditions consisted of 35 PCR cycles (1' 95°C, 2' 55°C, and 3' 72°C), followed by a single extension step of 10' at 72°C. Nucleotide sequences were determined on double stranded DNA, by the dideoxy-chain termination method (6).

Preparation of antigens and ELISA procedure

Extraction of LPS- and flagellar-antigens and the subsequent use of these antigens in an ELISA in order to detect S. enteritidis specific antibodies was as described previously (3).

Localization of the S. enteritidis H-type specific flagellar epitope

Construction and expression of recombinant-DNA clones, and the subsequent Western-blot assays on the recombinant-proteins encoded by these clones was performed essentially as described previously (7).

RESULTS

Validation of the flagellar antigens with sera from artificially infected chicks

Flagella purified from various Salmonella strains were tested for their specificity with both positive and negative control sera (data not shown). Flagella isolated from S. bergen reacted with sera from chickens infected with S. typhimurium. Flagella from S. godesberg reacted with sera from chickens infected with S. enteritidis, and with sera from chickens infected with S. agona. These results demonstrate that H-antigen specific antibodies are present in the sera in sufficient quantities to allow for a flagella based ELISA.

Comparison of lipopolysaccharide and flagellar antigens

The purified flagella were used to screen sera from commercial flocks. A total number of 335 randomly selected samples, each sample consisting of a pool of six sera was tested in an ELISA. A summary of the obtained results is presented in Table 1.

Comparison of ELISA results of the flagellar-antigen from S. godesberg with the LPS-antigen from S. enteritidis showed that 66% of the samples reacted similarly to both antigens, 75 samples were positive with both antigens, and 148 samples were negative with both antigens. Almost all of the remaining samples gave positive reactions to the flagellar-antigen and a negative reaction to the

LPS-antigen. Comparison of the ELISA data of S. godesberg flagella with those of S. bergen flagella showed that 86% of the samples gave similar reactions with both antigens. Of these, 44% gave positive reactions with both antigens and 56% gave negative reactions with both antigens

(Table 1). * only 287 of the 335 samples tested, @ lipopolysaccharide isolated from S. enteritidis was used as an antigen, # a mixture of lipopolysaccharide from S. typhimurium and S. panama, $ flagellar antigen isolated from S. godesberg, and + flagellar antigen isolated from S. bergen.

Amplification of the flagellin gene and sequence analysis.

Using primers derived from the S. enteritidis flagellin gene, and the DNA of heat denaturated S. enteritidis strain HK857 as template, a DNA fragment of about 1.5 kb was PCR-amplified and subsequently cloned in the multipurpose plasmid pBluescript (Stratagene, La Jolla, CA).

The nucleotide sequence of this DNA fragment was determined, and an open reading frame of 1479 bp could be identified in this sequence.

Hybridization experiments

Genomic DNA from S. enteritidis and S. typhimurium was digested with several restriction endonucleases, separated by agarose gel-electrophesis and blotted on Hybond-N (Amersham International, Amersham, UK). The filter was probed with a DNA-fragment encoding the conserved 5'-end of the cloned S. enteritidis flagellin gene. For all used restriction endonucleases the resulting autoradiograph showed a single hybridization signal with the digested S. enteritidis chromosomal DNA whereas S. typhimurium always gave two signals (data not shown). All hybridization signals were of the same intensity. This shows that, as opposed to S. typhimurium and many other Salmonella serotypes, the S. enteritidis chromosome contains only a single copy of the flagellin gene.

Characterization of the specificity of the monoclonal antibodies

The H-antigen specificity of the monoclonal antibodies (Mab) was determined by Western-blot analysis of these Mab against a panel of Salmonella strains (Table 2). From this analysis it was concluded that Mab 2 and 3 specifically recognized the flagellar g,m epitopes.

Localization of the H-type specific epitopes

To identify the potential serotype specific regions of the flagellin protein, the amino acid sequence of the S. enteritidis flagellin, as deduced from the determined nucleotide sequence, was aligned with published Salmonella flagellin sequences (data not shown). Based on restriction endonuclease information gathered from the nucleotide sequence and the location of potential serotype specific regions of the flagellin the following recombinant-DNA clones were constructed in the prokaryotic expression vector pEX: 12E3 representing the total flagellin, and KP-2, SS-23, SP-4, SS-28, SS-11, that encode a flagelin-fragment. The size and location of the proteins encoded by these clones is represented in Figure 1. The expression-products of these clones were purified and subsequently used in a Western-blot assay to determine their reactivity with the Mab as well as with a number of H-type specific polyclonal antibodies

(Table 3).# Mab recognition of the listed strains was assayed on Western-blots that contained total bacterial protein. +: flagellar protein is recognized by the Mab, -: no recognition.
* H-serotype according to Kauffman-White.

Figure 1. Location of recombinant-flagellin expression proteins. A:
schematic representation of the 1479 bp flagelin encoding PCR product. The location of the restriction endonuclease sites used in the construction of the recombinant-flagellin expression clones is indicated. B: The location of the encoded flagellin. The conserved (gray) and the variable area (white) as based on amino acid alignments are indicated. C: Relative location of the expression products encoded by the recombinant-DNA clones. The reaction of these expression products with the H-type g,m specific antibodies is indicated: white = negative, black = positive.

Tabel 3-* Mab: monoclonal antibody; α-g,m, α-g,m,s, and α-m,t: H-type g,m, g,m,s and m,t specific polyclonal antibodies, respectively.

Specificity of the recombinant flagellin protein

To establish whether the flagellar fragment expressed by clone SS-23 was specific for S. enteritidis the recognition of this recombinant protein by a panel of H-type specific polyclonal antisera was tested. The results of this ELISA are presented in Table 4.

Table 5 shows some preliminary ELISA results of the rec-DNA flagellin antigen with sera and egg yolk from experimentally infected chickens. The SS-23 antigen seems specific for those antisera that are derived from chickens that were infected with S. enteritidis. Furthermore, Table 5 shows that only eggs from chickens experimentally infected with S. enteritidis and not those from chickens infected with S. panama or S. typhimurium are positive in our SS-23 flagella-ELISA.

DISCUSSION

In our previous attempts to design an S. enteritidis specific ELISA significant cross-reactivity with the S. enteritidis LPS antigen was observed (8). The observed cross-reactivity probably due to O:12 antigen of this LPS. In order to circumvent this problem LPS isolated from members of the D2 group (O:9,46) was used. This proved to be of little value in reducing the cross-reactivity, as none of the D2 group LPS-preparations reacted with serum from chicks orally infected with S. enteritidis. This suggests that the O:12 antigen is immunodominant and therefore the O:9 antigen does not elicit a significant antibody titre.

Another antigen that can theoretically be used in an ELISA to discriminate between the different Salmonella serotypes is the flagella. To test the feasibility of a flagella-ELISA for the detection of S. enteritidis specific antibodies isolated flagella from S. godesberg and S. bergen were used. The flagellar proteins of these two serotypes have the same H antigen as S. enteritidis and S. typhimurium, respectively, but the LPS of these two Salmonella serotypes carries different O antigens than S. enteritidis. ELISA-titre of chicken-sera against the purified flagellar antigens shows that sufficient antibody titre against flagella exist to allow for a flagella-ELISA. This study shows that flagellar antigens give specific reactions with sera from orally infected chicks. Reactions seem to be specific for the H antigens present in the flagella of these serotypes, as flagellar antigen from S. godesberg reacts both with sera from S. enteritidis and S. agona infected chickens.

When the flagella isolated from S. godesberg and S. bergen were used to screen sera derived from chickens of commercial flocks, a significant cross-reactions with these flagellar antigens was observed. Of the 335 serum samples 127 gave positive reactions on both of the flagellar antigens tested, H:g,m and H:i, indicating they share 'common' epitopes (Table 1). The occurrence of this cross-reactivity in 'field-sera' was not surprising since it was reported (9) that there are immunological relationships, not only between flagellins of different Salmonella serotypes but also between these and flagellins from other Enterobacteriacea. It was suggested (10,11) from comparisons of the protein sequences deduced from the DNA-sequence of the flagellin genes of several Salmonella strains (belonging to different H-serotypes) that these cross-reactive common epitopes are most likely situated in the N- and C-terminus of the flagellin gene, whereas serotype specific antigens (like those used in the Kaufmann-White scheme) probably reside in the middle.

It was demonstrated that it was possible to distinguish between sera from chickens artificially inoculated with different Salmonella serotypes using purified flagella as a capture antigen. This indicates that a significant immune response to these serotype specific antigens must exist. Thus if the common epitopes of these flagella could be removed they would probably result in the desired specific capture antigen.

With Southern blots it was demonstrated that S. enteritidis carries only a single flagellin gene. The S. enteritidis flagellin gene was PCR amplified and sequenced and the encoded amino acid were compared with previously published flagellins of various Salmonella serotypes. Based on this information recombinant-DNA clones were constructed in an expression vector, each clone expressing a different part of the flagellin (Figure 1). Using these clones and a panel of g,m-specific Mab's (Table 2) the precise location of S. enteritidis H type specific epitopes was determined (Table 3).

The smallest fragment that still encoded these epitopes was subsequently expressed and the purified expression-product was used as antigen in an ELISA. The obtained data show that this results in an indirect ELISA that can be used for demonstration of the presence of antibodies against Salmonella enteritidis in both sera and in eggs. The resulting ELISA is a simple to execute and fast technique to trace antibodies against the Salmonella H-type gm. Large scale infection experiments and a the testing of a large number of well documented field sera is currently being performed. The collected data will be compared data obtained from other tests. Although this testing is far from complete, the preliminary results indicate that all flocks that were found positive with our SS-23 based flagella-ELISA were also found positive with other tests (LPS-based ELISA and bacterial cultures from faeces). However, if the data on individual chicken were compared both negative and (highly) positive individuals were found present in the same population. When looking at the level of the individual chicken, comparing the titres from individual chickens in SS-23 and LPS based ELISA titres did not show an obvious correlation. All possible combinations could be found; i.e. both tests positive, both tests negative, or one test negative whit the other clearly positive. The observed antibody titres against flagella and LPS may not be related, since there are many factors that influence these titre, both in respect to the time and route of exposure to bacterium, as well as to the different stages of maturation of the immune system. Preliminary results (data not shown) indicate that the antibody titre to flagellar antigens reach high levels shortly after infection, while LPS titre usually develop slower. On the other hand, once the infection has ended, flagellar antibody titre tend drop faster than LPS titre. These preliminary data indicate that flagellar antigens might be a better candidate in an ELISA than LPS, as both fewer false-negatives, due to a slow response, and fewer false-positives, due to long-lasting antibody levels will occur, as compared to LPS. The obtained data merit further research to test the above assumptions.

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