Serological response of sheep to plasmid-encoded proteins of *Yersinia* species following natural infection with *Y. enterocolitica* and *Y. pseudotuberculosis*

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**Summary.** A prospective study of the serological response to natural infection with *Yersinia enterocolitica* and *Y. pseudotuberculosis* was performed in an experimental flock of sheep. A preliminary investigation with immunoblotting techniques showed that lambs infected with virulent *Yersinia* spp. produced antibodies to several yersinia outer-membrane proteins (yops) encoded by a virulence plasmid (pYV) of *Yersinia enterocolitica* or *Y. pseudotuberculosis*. Thereafter, an enzyme immunoassay (EIA) was developed to measure antibodies to yops. Criteria for interpreting the EIA were established by examining sera from a negative control population of lambs which had not been infected with *Yersinia* spp. since birth. Test samples comprised 25 pairs of pre- and post-infection sera from animals with bacteriologically proven infections with *Yersinia* spp. The results showed that infection of lambs with pYV-bearing strains of *Y. enterocolitica* or *Y. pseudotuberculosis* invariably evoked a significant antibody response to yops, even though all the infections were subclinical. No animal infected with so-called "environmental", pYV-negative *Yersinia* spp. seroconverted to yops. EIA with yops as antigen provided a sensitive and specific means to diagnose subclinical infection of lambs with virulent *Yersinia* spp.

**Introduction**

The enteropathogenic *Yersinia* species, *Y. enterocolitica* and *Y. pseudotuberculosis*, infect a wide range of animals, including man. Although the species and bio-serogroups of *Yersinia* that infect various animal species may differ, all virulent *Yersinia* carry a virulence plasmid of ~ 70 kb termed pYV. This plasmid encodes the synthesis of a family of *yersinia* outer-membrane and secreted proteins (yops), some of which make a direct contribution to pathogenicity. Virulence plasmids obtained from *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* show extensive DNA homology and the yops encoded by them are antigenically related. When pYV+ strains of *Y. enterocolitica* and *Y. pseudotuberculosis* are cultivated under appropriate conditions in vitro (i.e., at 37°C in a medium containing a reduced concentration of calcium ions), several yops are secreted into the culture medium. These proteins are also produced during the course of infection, because sera obtained from human patients and experimental animals convalescing from infection with *Yersinia* spp. commonly contain antibodies to them. These observations led to the development of serological assays with yops as antigens to diagnose infection with *Yersinia* spp. in man. Evaluation of these assays in patients with bacteriologically proven infections with *Y. enterocolitica* and *Y. pseudotuberculosis* have shown them to be sensitive and specific. Investigations of this type, however, are necessarily retrospective, and provide only indirect evidence of seroconversion.

To determine the sensitivity and specificity of yop-based serological tests, a prospective study was performed of natural infections with *Yersinia* spp. in sheep and lambs in the Gippsland region of Victoria, Australia. The availability of serial faecal and serum samples from lambs infected with pYV+ and pYV- strains of *Yersinia* spp. allowed the establishment of a convenient serological assay for yop-specific antibodies and the determination of criteria for seroconversion to yops and for serological evidence of recent infection with virulent *Yersinia* spp.

**Materials and methods**

**Animals**

The conditions under which the experimental flock of ewes and lambs was maintained have been described previously. Briefly, the flock was maintained...
at the Regional Veterinary Laboratory of the East Gippsland Agricultural Centre and comprised 20 7-year-old crossbred ewes and their offspring. Lambs were born between late June and early November 1989, and between mid-July and early August 1990. They were weaned at 8–12 weeks of age by separating them from their mothers for 2–3 weeks. The flock was grazed in paddocks that had held sheep, goats and cattle in previous years. Three merino sheep that were excreting pYV+ Y. enterocolitica were introduced to the flock in February 1990. No infections with pYV+ Y. enterocolitica occurred before this time. The period for which individual animals were studied ranged from 3 to 18 months. All animals that became infected with Yersinia spp. during the course of the study did so as a result of natural infection.

Microbiological methods

Rectal swabs were collected from each animal at approximately weekly intervals and inoculated on to Yersinia Selective Agar (CIN, Oxoid). Plates were incubated for 40 h at 30°C. Yersinia spp. were identified by standard biochemical tests and assigned to a biotype. Strains were examined for carriage of pYV by culturing them on MOX agar at 25°C and 37°C.9

Sera

Blood for serological analysis was drawn from a jugular vein of most animals at 1- or 2-month intervals and allowed to clot at room temperature. The serum was removed and stored at 4°C until required. Of 31 lambs investigated, 12 remained culture-negative for Yersinia spp. during the entire course of the study. Accordingly, paired pre- and post-infection sera were available from 19 animals. Two lambs were infected at different times, almost 1 year apart, with Y. pseudotuberculosis and Y. enterocolitica. Four lambs were infected sequentially with pYV- and pYV+ strains of Yersinia of different species or biotypes. Thus, a total of 25 serum pairs was available for study.

Nine pairs of sera were from lambs with a bacteriologically proven infection with a pYV- strain of Y. enterocolitica. These bacteria belonged to various serogroups, but all were biotype 1A. Seven serum pairs were from animals naturally infected with P-yV+ Y. enterocolitica, all of which were O-serogroup 2,3, biotype 5. Nine pairs of sera were from lambs in which pYV+ strain of Y. pseudotuberculosis was the only pathogen identified. All these isolates belonged to serogroup III.

Preparation of secreted yops

Y. enterocolitica 30.42.67 (O-serogroup 3, biotype 4) and Y. pseudotuberculosis 1897 (O-serogroup III) were pYV+ strains isolated from the faeces of a patient in Sweden and a deer in New Zealand, respectively. Y. pseudotuberculosis 1897 was kindly provided by B. Buddle, Wallaceville Animal Research Centre, Upper Hutt, New Zealand.

Secreted yops were prepared by growing these bacteria overnight at 25°C in Brain Heart Infusion Broth (Grand Island Biological Company, Grand Island, NY, USA) containing glucose 0.4% w/v (BHIB-G). This suspension (0.5 ml) was inoculated into 10 ml of BHIB-G containing 20 mM MgCl2 and 20 mM sodium oxalate. Bacteria were cultivated with shaking at 25°C for 2 h. The incubation temperature was then raised to 37°C and incubation was continued for a further 4 h. Bacteria were removed by centrifugation at 8000 g for 10 min. Solid ammonium sulphate (4.76 g) was added slowly to the supernate and the proteins were precipitated overnight at 4°C. The precipitate was pelleted by centrifugation at 1900 g and resuspended in 150 µl of sample buffer SDS 4.5% w/v, β-mercapto-ethanol 7.5% v/v, bromophenol blue 0.0075% w/v, 0.075 M Tris-HCl, pH 6.8). The final protein concentration was adjusted to 50 µg/ml (BioRad protein assay, BioRad, Richmond, CA, USA). For the enzyme immunoassay, the precipitate was dissolved in carbonate-bicarbonate coating buffer (15 mM Na2CO3, 35 mM NaHCO3, 3 mM NaN3, pH 9.6), at a final protein concentration of 5 µg/ml. Control material prepared from a pYV- derivative of Y. enterocolitica 30.42.67 was used to examine the specificity of the serological assays.

Immunoblotting

Secreted yops in 150 µl of sample buffer were boiled for 5 min and 20 µl of this solution, containing 1 µg of protein, were loaded into each well of a polyacrylamide gel, comprising acrylamide 10% w/v. Electrophoresis was conducted at 15 mA for 6-5 h. Separated proteins were then transferred to a 0.45-µm pore size nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) by electroblotting at 20 V for 30 min in a solution of 49 mM Tris, 39 mM glycine, 1 mM methanol 20% v/v, 0.05 M Tris-HCl, pH 7.4. The final protein concentration was adjusted to 50 µg/ml by phenol blue 0.0075% w/v and resuspended in blocking solution of skimmed milk powder. Membranes were incubated with gentle agitation at 4°C overnight in a blocking solution of skimmed milk powder 5% w/v in Tris buffered saline (TBS), comprising Tween 20 0.05% v/v, 0.05% Tris-HCl, 0.15 M NaCl, pH 7.4.}

Enzyme immunoassay (EIA)

Yops (0.5 µg) in 100 µl of carbonate-bicarbonate coating buffer were dispensed into each well of a 96-well
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Fig. 1. Immunoblot of yops prepared from \textit{Y. pseudotuberculosis} (lanes 1–4), and \textit{Y. enterocolitica} (lanes 5–8). Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and treated with pre-immune (lanes 1, 3, 5, 7) and immune (lanes 2, 4, 6, 8) sera from lambs with culture-proven infection with pYV\textsuperscript{+} \textit{Y. enterocolitica} (lanes 1, 2, 5, 6) and \textit{Y. pseudotuberculosis} (lanes 3, 4, 7, 8). The positions of yops from \textit{Y. pseudotuberculosis} and \textit{Y. enterocolitica}, and their mol. wts determined from the mobility of pre-stained standards, are shown on the left and right of the figure, respectively.

Maxisorp Immuno-plate (Nunc, Roskilde, Denmark) and left at 4°C overnight. Plates were washed four times in PBS-Tween buffer, comprising 137 mM NaCl, 8 mM KCl, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 8 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4. One hundred \(\mu\)l of each test serum, diluted 1 in 1000 in PBS-Tween containing casein 12 mg/ml, were added to each well and incubated at 37°C for 1 h. Plates were washed four times in PBS-Tween buffer, after which 100 \(\mu\)l of donkey anti-sheep IgG-peroxidase conjugate, diluted 1 in 1000 in PBS-Tween-casein, were added to each well. Plates were incubated for 1 h at 37°C, washed four times, and 100 \(\mu\)l of peroxidase substrate (Kirkegaard and Perry Lab. Inc., Gaithersburg, MD, USA) were added to each well and left at room temperature until colour developed. The reaction was stopped by the addition of 2 M sulphuric acid and the plates were read in a Diagnostics Pasteur LP400 plate reader (Sanofi, Marnes-la-Coquette, France) at 450 nm. Results were expressed as the mean net OD (after subtraction of the blank reaction) of duplicate wells assayed on at least two separate occasions.

\textbf{Data analysis}

Data were analysed by paired or unpaired two-tailed Student’s \(t\) test as appropriate. A critical \(p\) value of 0.05 was used for all analyses.

\textbf{Results}

\textbf{Immunoblotting}

In a preliminary study of the immune response of lambs to individual yops from \textit{Y. enterocolitica} and \textit{Y. pseudotuberculosis}, serum was examined from two animals that had been infected with pYV\textsuperscript{+} \textit{Y. pseudotuberculosis} and \textit{Y. enterocolitica}, respectively. The results showed that seroconversion occurred to as many as six separate yops from \textit{Y. pseudotuberculosis} and to eight from \textit{Y. enterocolitica} (fig. 1). Both convalescent sera recognised the same medium–low mol.-wt (26–49 kDa) proteins in the two yop preparations, regardless of whether the animals had been infected with \textit{Y. pseudotuberculosis} or \textit{Y. enterocolitica}.
(fig. 1). However, these sera did react more strongly, with high mol-wt (65-88 kDa) yops from the homologous species (fig. 1). None of the sera reacted with material prepared from the pYV- derivative of Y. enterocolitica 30.42.67 (data not shown).

**Enzyme immunoassay (EIA)**

In preliminary evaluation of the EIA, yops from Y. enterocolitica were used as antigen to examine serum samples from 38 lambs in this study whose faeces had not yielded Yersinia spp. since birth. The ages of these animals at the time of sampling were 3-12 months. The results of these negative control sera showed a mean net OD of 0.051 (SD 0.049). These data were used to establish criteria for (i) a positive titre and (ii) for seroconversion, which were defined respectively as (i) a net OD value ≥ 2 SD above the mean negative value, and (ii) an increase in net OD of > 2 SD. Application of the latter value to paired sera from lambs with bacteriologically proven yersiniosis is illustrated in fig. 2. None of nine lambs infected with pYV- strains of Y. enterocolitica showed a serological response to yops. The mean net OD for these animals before infection was 0.06 (SD 0.03) and after infection was 0.04 (SD 0.02), p > 0.2 (table). In contrast, all seven lambs infected with a pYV+ strain of Y. enterocolitica showed a pronounced serological response, with a mean increase in net OD of 0.84 (SD 0.23), p < 10^-4. Lambs infected with Y. pseudotuberculosis also showed a highly significant increase in antibody titre following infection (mean increase in net OD 0.63, SD 0.37, p < 10^-4). Although the increase in titre of these sera was less than that in sera from animals infected with Y. enterocolitica, this difference was not significant (p > 0.2).

The EIA was repeated with yops from Y. pseudotuberculosis as antigen. Although this assay was as sensitive and specific as that with yops from Y. enterocolitica, the net ODs of the negative control sera were higher and the post-infection values lower than in the EIA with yops from Y. enterocolitica (fig. 2B, table). These findings can be explained in part by the finding on immunoblotting that sera from animals infected with pYV+ Yersinia spp. recognised more yops from Y. enterocolitica than from Y. pseudotuberculosis (fig. 1).

**Table.** Antibody response to yops from Y. enterocolitica and Y. pseudotuberculosis in sera from sheep infected with Yersinia spp.

<table>
<thead>
<tr>
<th>Strain isolated from faeces (number of animals)</th>
<th>Y. enterocolitica</th>
<th>Y. pseudotuberculosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before infection</td>
<td>After infection</td>
<td>Before infection</td>
</tr>
<tr>
<td>pYV- Y. enterocolitica (9)</td>
<td>0.06 (0.03)</td>
<td>0.16 (0.08)</td>
</tr>
<tr>
<td>pYV+ Y. enterocolitica (7)</td>
<td>0.08 (0.04)</td>
<td>0.17 (0.14)</td>
</tr>
<tr>
<td>pYV+ Y. pseudotuberculosis (9)</td>
<td>0.04 (0.03)</td>
<td>0.13 (0.04)</td>
</tr>
</tbody>
</table>
Discussion

This investigation has confirmed the value of EIA with yops as antigen in the serodiagnosis of infections with pathogenic strains of \textit{Y. enterocolitica} and \textit{Y. pseudotuberculosis}. All 16 lambs infected with pYV+ strains of \textit{Yersinia} spp. seroconverted to yops, whereas uninfected lambs did not. Thus, the demonstration of seroconversion was 100\% predictive for infection with virulent \textit{Yersinia} spp. EIIAs with yops from \textit{Y. enterocolitica} and \textit{Y. pseudotuberculosis} as antigen were equally sensitive and specific, regardless of the species responsible for the infection.

One clear advantage of serological assays based on yops is that they eliminate the need for the battery of antigens required for tests based on O-antigens. As the number of O-serogroups of \textit{Yersinia} that infect man and animals is legion, few laboratories can afford to perform exhaustive serological analyses with tests based on three antigens. Furthermore, the fact that some O-antigens of \textit{Yersinia} spp. cross-react with those of other bacteria, may confound interpretation of these assays. However, the finding of yop-specific antibodies provides no information as to the species or serogroup of the infecting strain. If such information is required, tests for O-specific antibodies should be conducted.

Although the lambs in this study that were infected with virulent \textit{Yersinia} spp. showed a pronounced antibody response to virulence antigens, none of them demonstrated overt evidence of infection. However, the fact that all of these animals seroconverted is a clear indication of active infection involving bacterial replication \textit{in vivo}, because yersiniae do not produce yops when cultivated under environmental conditions, where temperatures generally are below 30°C and calcium ion concentrations are high. The presence of anti-yop antibodies in animals with subclinical infections indicates that EIA may be useful in seroepidemiological surveys of yersiniosis in various animal species. EIA could also be used to screen food animals, such as sheep and pigs, for \textit{Yersinia} spp., and possibly even as a component of plague surveillance in rats. However, samples that gave a positive result under the latter circumstances would need to be confirmed with a species-specific antigen, because of the possibility of a false-positive result in rats infected with a pYV+ strain of \textit{Y. pseudotuberculosis} or \textit{Y. enterocolitica}.

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References