Analysis of saliva for antibodies to the LPS of *Escherichia coli* O157 in patients with serum antibodies to *E. coli* O157 LPS

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The salivary antibody response to the *Escherichia coli* O157 LPS antigen was assessed in 44 patients with serum antibodies binding to the LPS of *E. coli* O157. Saliva from 477 controls was also examined to assess the specificity of the immunoassay used. Twenty of the 44 patients had salivary antibodies to *E. coli* O157 LPS, giving the salivary antibody test a sensitivity of 0.45 and a predictive positive value for seropositivity of 1.00. The presence of these antibodies appeared not to relate to the time interval between serum sampling and saliva sampling. None of the 477 volunteers had salivary antibodies binding to the LPS of *E. coli* O157 alone; however, 15 had antibodies which bound non-specifically to both O157 LPS and BSA.

INTRODUCTION

Verocytotoxin-producing *Escherichia coli* (VTEC) of serotype O157:H7 and O157:H– continue to be a cause of bloody diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome (HUS) in the UK (Simmons, 1997). Patients infected with *E. coli* O157 produce serum antibodies to the O157 LPS antigens (Chart & Jenkins, 1999), and these antibodies form the basis for routine serological tests providing evidence of infection in the absence of faecal *E. coli* O157 (Thomas *et al.*, 1996). The kinetics of serum antibody production during pathogenesis of infection with *E. coli* O157 has not been investigated thoroughly due to a paucity of sequential sera from patients. However, a recent study involving a single patient showed that the primary response involved high levels of IgM-class antibodies to O157 LPS, peaking at 10 days post onset of disease. Reduction in levels of IgM-class antibodies coincided with the emergence of IgG-class antibodies to O157 LPS (Chart *et al.*, 2002). In general, sera used for O157 serodiagnosis originate from samples of blood taken for blood biochemistry, and the time of blood sampling may not coincide with maximal levels of antibodies to *E. coli* O157 LPS. Additional blood samples tend not to be taken, and if an initial serum did not contain antibodies to *E. coli* O157 LPS, the cause of disease may go undetected.

Patients infected with *E. coli* O157 may also produce salivary antibodies to the O157 LPS antigen (Chart & Jenkins, 1998), and the ease of taking samples of saliva would make this very

suitable for serodiagnosis, particularly for young children where taking samples of blood may be problematical. Analysis of sequential saliva samples would also increase the likelihood of testing patients when levels of antibodies were optimal. In the present study, 44 patients with serum antibodies to *E. coli* O157 LPS were examined for salivary antibodies with an O157-LPS-based ELISA combined with an immunoblotting procedure. Saliva from 477 controls was used to determine the likelihood of the immunoassay giving false-positive reactions.

METHODS

Patients. Forty-four patients were involved in this study (Tables 1 and 2) and all were shown to have serum antibodies to the LPS of *E. coli* O157 by routine serological analysis (Chart & Jenkins, 1999). Twenty of the patients were female (mean age 27.8 years, $\sigma = 23.4$ years, $n = 13$) and 13 male (mean age 18.3 years, $\sigma = 15.5$ years, $n = 9$).

Symptoms included HUS (9), haemorrhagic colitis (1), diarrhoea (3) and bloody diarrhoea (1). Patients in whom serum antibodies to *E. coli* O157 were detected were requested to provide saliva samples; the time interval between the serum date and saliva date was calculated where a date of saliva sampling was provided (Tables 1 and 2). On receipt, sera were stored at -30°C until tested.

Saliva sampling. Samples of saliva were obtained using Oracol saliva test kits (Malvern Medical Developments). These comprised a sponge attached to a stick, enclosed within a plastic transport tube. The sponge–stick arrangement was used like a toothbrush, with the aim of absorbing saliva from both the mouth and the gingival crevices around the teeth. On arrival in the laboratory, the swabs were maintained at 4°C until required for testing. Sponges were then detached, placed in a 5 ml syringe and the saliva was pressed from the sponge.

Abbreviations: HUS, haemolytic uraemic syndrome; VTEC, verocytotoxin-producing *Escherichia coli*.

Table 1. Patients with serum and salivary antibodies to *E. coli* O157 LPS

No.	Time (days)*	Properties of VTEC O157 where isolated†
61	38	
03	28	PT 2 VT2
53	27	PT 21/28 VT2
05	27	PT 21/28 VT2
42	26	PT 21/28 VT2
33	23	
60	15	PT 21/28 VT2
59	15	PT 21/28 VT2
58	15	PT 21/28 VT2
57	15	PT 21/28 VT2
54	15	PT 21/28 VT2
50	15	PT 21/28 VT2
07	13	
34	5	PT 38 VT2
24	?	PT 2 VT2
17	?	PT 14 VT2
08	?	
01	?	
06	?	PT 2 VT2
26	?	

*Time in days between serum sample and saliva sample; ?, time between serum sample and saliva sample were not known.

†PT, phage type; VT, verocytotoxin type.

Volunteers. Samples of control saliva were obtained from 52 members of PHLS staff and their families; 23 were female (mean age 29.2 years, $\sigma = 17.4$ years, $n = 23$) and 19 male (mean age 30.3 years, $\sigma = 15.75$ years, $n = 19$). Samples of control saliva were also obtained from 425 children, under the age of 11 years, attending a Live Science exhibition at the Science Museum in London. The children comprised 173 girls (mean age 7.8 years, $\sigma = 2.1$ years) and 252 boys (mean age 7.7 years, $\sigma = 2.0$ years). Samples of saliva were screened by ELISA and those giving values $> 0.5 A_{405}$ or equivocal results were tested by immunoblotting.

Isolation and characterization of VTEC O157. Twenty-three of the 44 patients in the study had presumptive VTEC O157 isolated from their stools by clinical laboratories. These isolates were confirmed biochemically, serotyped, phage-typed and tested for the presence of verocytotoxin-encoding genes by the method summarized by Willshaw *et al.* (2001).

LPS. For SDS-PAGE and immunoblotting, LPS was prepared by digesting whole bacteria with proteinase K (Chart *et al.*, 1989a). Bacteria were placed in pre-weighed Eppendorf tubes and the cells suspended in SDS-PAGE sample buffer (Laemmli, 1970) to give a concentration of 1 mg per 30 μ l prior to incubation at 100 °C for 10 min. After cooling, samples were mixed with an equal volume of SDS-PAGE buffer containing 100 μ g proteinase K (Sigma) per 30 μ l prior to incubation at 60 °C for 1 h.

For the ELISA, LPS was prepared from outer membranes (Chart, 1994) based on the hot-phenol method of Westphal & Jann (1965). Outer membranes were suspended in 5 ml deionized water and mixed with

Table 2. Patients with serum but not salivary antibodies to *E. coli* O157 LPS

No.	Time (days)*	Properties of VTEC O157 where isolated†
22	44	
82	39	PT 49 VT2
63	32	
80	30	
4	23	PT 21/28 VT2
2	16	
52	15	PT 21/28 VT2
55	15	PT 21/28 VT2
51	15	PT 21/28 VT2
75	14	
10	14	
48	12	PT 21/28 VT2
36	7	PT 8, VT1 and 2
77	?	PT 2 VT2
114	?	PT 21/28 VT2
27	?	
25	?	
81	?	
100	?	
102	?	
73	?	
123	?	
101	?	
79	?	

*Time in days between serum sample and saliva sample; ?, time between serum sample and saliva sample were not known.

†PT, phage type; VT, verocytotoxin type.

5 ml 80% (w/v) aqueous phenol solution (Merck). Following incubation at 68 °C for 15 min, the preparation was centrifuged (3000 g, 30 min, RT) and the aqueous phase was harvested and transferred to a clean glass container. The remaining phenol phase was mixed with 5 ml deionized water, incubated at 68 °C for 15 min and centrifuged (as above). The aqueous phase was removed and mixed with the initial aqueous phase fraction prior to dialysis against 3×5 l deionized water at 4 °C. The aqueous fraction was lyophilized and the LPS was weighed. The LPS was analysed by SDS-PAGE alongside LPS prepared from whole bacteria (see above). Profiles were stained with a silver stain for carbohydrate (Tsai & Frasch, 1982) and showed that the purified LPS was representative of whole-cell LPS. They were also stained with a silver stain for protein (Wray *et al.*, 1981) and shown not to contain contaminating cellular proteins.

SDS-PAGE and immunoblotting. SDS-PAGE was performed using an Atto mini-gel apparatus (Genetic Research Instruments). Preparations containing 83 μ g digested cell mass were used per lane, loaded onto gels (4.5% stacking gel and 12.5% separation gel) and electrophoresis was performed (50 mA) for 30 min. Gels were either stained with silver to show LPS profiles (Tsai & Frasch, 1982) or with Coomassie blue for proteins (Chart *et al.*, 1989a), or profiles were transferred onto nitrocellulose paper (NCP) by immunoblotting (Chart *et al.*, 1989a) (0.50 A, 1 h). Profiles immobilized on sheets of NCP were blocked with

3 % skimmed milk in PBS (milk-PBS; 30 min) and reacted with 30 µl serum in 5 ml milk-PBS for 60 min. After washing in PBS-Tween (3 × 10 min), profiles were reacted with 5 µl per lane of goat anti-human polyvalent Ig conjugated with alkaline phosphatase (Sigma) in skimmed milk-PBS (60 min). After washing, as above, profiles were placed in substrate buffer (0.1 M Tris, 0.09 M NaCl, 0.15 M MgCl₂ · 6H₂O) for 10 min. For colour development, NCP strips were placed in substrate buffer (10 ml) containing 45 µl nitro blue tetrazolium (Sigma; 75 mg ml⁻¹ in 70 % aqueous dimethyl formamide) and 35 µl 5-bromo-4-chloro-3-indolylphosphate · Na₂ (Sigma; 50 mg ml⁻¹ in deionized water).

ELISA. The ELISA was performed (Chart *et al.*, 1989a) using O157 LPS. Each sample of saliva was reacted with duplicate wells 'coated' with 1 µg LPS in 100 µl coating buffer (1.59 g Na₂CO₃ and 2.93 g NaHCO₃ l⁻¹, pH 9.6) and 'blocked' with PBS containing BSA (10 g l⁻¹; Sigma). Samples were also reacted with a duplicate set of wells which had been blocked only. Samples of saliva (50 µl per well) were added and antibody binding was detected with the same anti-human antibody conjugate as described for immunoblotting (above), diluted 1 in 1000 in PBS (50 µl per well). Antibodies conjugated with alkaline phosphatase were detected by adding 200 µl diethanolamine buffer (1 M diethanolamine, 2 mM MgCl₂, pH 9.6) containing 1 mg *p*-nitrophenol phosphate ml⁻¹ (Sigma). The resultant colour was quantified by measuring the A₄₀₅. Samples with ELISA values of > 0.5 A₄₀₅ or equivocal results were examined by immunoblotting (Chart *et al.*, 1989b).

RESULTS AND DISCUSSION

Of the 44 patients with serum antibodies to O157 VTEC LPS, 20 (45.5 %) also had salivary antibodies binding to O157 LPS as shown by immunoblotting (Table 1). This proportion was lower than observed in an earlier study (Chart & Jenkins, 1998) where 62 % of patients were identified with O157-LPS-specific salivary antibodies. The remaining 24 patients did not have detectable levels of salivary antibodies to O157 LPS, and the reasons for this remain unknown. The sensitivity of the salivary antibody test was calculated to be 45 %, with a predictive positive value of 100 %. This was considerably lower than the values obtained by Ludwig *et al.* (2002), who observed sensitivities of 100 % and 92 %, respectively, for IgM- and IgG-class antibodies. The study by Ludwig *et al.* (2002) had the advantage of analysing sequential saliva samples from patients suspected of having been infected with *E. coli* O157. Regrettably, this was not possible in the present study where only single samples of saliva were obtained.

Of the 20 patients with salivary antibodies to O157 LPS, strains of VTEC O157 were isolated from 14 (70 %). These carried the genes encoding VT2 and nine belonged to PT 21/28 (Table 1). VTEC O157 was isolated from nine (37.5 %) of the remaining 24 patients with eight of nine strains carrying genes encoding VT2 and six belonging to PT 21/28 (Table 2). It was interesting to note that O157 VTEC was isolated from almost twice as many patients with salivary antibodies to O157 LPS as compared to those who did not have these antibodies, but the reasons for this are not known.

For the 20 patients with salivary antibodies to O157 VTEC LPS, the time intervals between submitting antibody-positive serum samples and the detection of anti-O157

antibodies in saliva samples ranged from 5 to 38 days (Table 1). For patients without salivary antibodies to O157 VTEC LPS, the time intervals between submitting antibody-positive serum samples and the receipt of saliva samples ranged from 7 to 44 days (Table 2). The time intervals between submitting serum samples and saliva samples, for patients with and without salivary antibodies to O157 LPS, were compared and found not to be significantly different ($\rho = 0.05$). From this it was concluded that a proportion of serum-antibody-positive patients may simply not produce salivary antibodies to O157 LPS during infection with VTEC O157. Unfortunately, the study was hampered by the absence of clinical information relating to the time interval between onset of infection and the time of blood and saliva sampling.

The study also examined samples of saliva from two control groups. Based on an ELISA cut-off value of 0.5 A₄₀₅, none of the 52 PHLS volunteers had salivary antibodies binding to the LPS of *E. coli* O157 alone; however, eight (mean age 27.6, $\sigma = 12.5$, $n = 5$) had salivary antibodies which bound non-specifically to both O157 LPS and the ELISA plate blocking protein, BSA. Saliva samples from these eight were shown not to contain antibodies binding to O157 LPS by blotting.

Similarly, by ELISA none of the 425 children under the age of 11 years had salivary antibodies to O157 LPS, demonstrating that the immunoassays in this study were unlikely to produce false-positive results. Seven (mean age = 7.4 years, $\sigma = 1.6$ years, $n = 7$) had salivary antibodies which also bound non-specifically to both O157 LPS and BSA. The reasons for this are not known, but this result illustrated the importance of incorporating the appropriate controls in immunoassays of the type used in the present study. From the results obtained here and by others (Ludwig *et al.*, 2002), and considering the detection of salivary antibodies to *E. coli* O157 LPS does not involve an invasive procedure, we suggest that clinicians investigating suspected cases of infection caused by *E. coli* O157 should consider having sequential samples of saliva analysed for antibodies to *E. coli* O157 LPS.

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