The distribution of, and antibody response to, the core lipopolysaccharide region of *Escherichia coli* isolated from the faeces of healthy humans and cattle

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INTRODUCTION

Lipopolysaccharide (LPS: endotoxin) is an integral part of the outer membrane of all Gram-negative bacteria. The complete (smooth) LPS structure of *Escherichia coli* and related species may be divided into three distinct regions: (i) the hydrophobic lipid A moiety, responsible for the endotoxic properties of LPS (Galanos et al., 1985); (ii) the core oligosaccharide, of which there are five known *E. coli* core types denoted R1, R2, R3, R4 and K12 comprising three 3-deoxy-D-manno-2-octulosonic acid (KDO) and three heptose residues along with five or six variable sugars: these define the core type (Amor et al., 2000) and (iii) the O-polysaccharide antigen which is easily accessible to the host immune system and leads to the generation of O-specific immune responses, thus the term O-antigen is commonly used (Erridge et al., 2002).

There are over 170 O-antigen serotypes recognized in *E. coli*, yet only a few have been implicated in disease. These include O1, O2, O4, O6, O7, O8, O15, O18 and O75 which are most often associated with blood-borne and urinary tract infection. Other types are more often associated with diseases of the gastrointestinal tract and include O15, O26, O55, O86, O91, O111 and O157 (Ørskov & Ørskov, 1974; Gibb et al., 1992). The core types associated with the former group are R1 and R2, while those with the R3 core are usually associated with the G1 pathogens (Gibb et al. 1992; Currie & Poxton, 1999; Amor et al., 2000 and I. R. Poxton, unpublished data). The cores of LPS from other organisms, for example *Salmonella* and *Shigella* spp. are identical to those of *E. coli* (Currie et al., 2001).

An immune response to the O-polysaccharide is usually considered to be protective, but serotype specific. However, a response to the core may be protective to a range of different serotypes. This has been proved by the use of cross-reactive, cross-protective anti-LPS core monoclonal antibodies (di Padova et al., 1993), and is strongly suggested by increased protection to endotoxic sequelae in patients with naturally high levels of anti-LPS core antibodies prior to surgery (Bennett-Guerrero et al., 1997). Overall, however, the response to whole LPS must be considered as the sum of the responses to both the core and the O-antigen (Currie et al., 2001).

There are five different core types of *Escherichia coli* lipopolysaccharide (LPS), and enterohaemorrhagic *E. coli* tend to have the R3 core type. It has been hypothesized that increased carriage of bacteria with a specific core type will induce higher levels of antibodies and protect against disease caused by bacteria carrying that specific LPS core. Approximately 320 isolates of *E. coli*, half from healthy human faeces and half from healthy bovine faeces have been core typed both by core-specific monoclonal antibodies, and by PCR for genes encoding the enzymes responsible for the biosynthesis of the specific core structures. Results showed that *E. coli* possessing R1 core LPS were most frequently detected in both human and cattle populations (63 and 49 %, respectively). Compared to the human isolates a significantly higher level of bacteria with R3 core LPS was detected among the bovine commensal *E. coli* (11 % compared to 4 %; *P* < 0.05).

Antibody levels to each of the specific core types were measured in serum samples from healthy humans (*n* = 91) and healthy cattle (*n* = 39). In each population the highest level of antibody detected was reactive to the R4 core. In cattle the level of anti-R3 core antibody was significantly higher than the level of anti-R1, -R2 and -K12 antibodies (*P* < 0.01). In summary there was a greater proportion of *E. coli* with R3 core type in cattle, together with a corresponding higher anti-R3 antibody level. This suggests that cattle may have greater immunity to *E. coli* strains with an LPS of R3 core type.

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; LPS, lipopolysaccharide; VTEC, verotoxin-producing *Escherichia coli*. 

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We have hypothesized that increased carriage of bacteria with a specific core type will induce higher levels of antibodies and protect against disease caused by bacteria carrying that specific LPS core – either at the mucosal level or systemically.

E. coli O157 : H7 and other enterohaemorrhagic E. coli (EHEC) strains are now established as major human pathogens implicated in a number of large disease outbreaks. Cattle are a known reservoir for E. coli O157 and other EHECs, yet disease in cattle is not widely recognized. It is unclear what role, if any, the bovine immune response plays in protection against EHEC-associated disease, but it is possible that antibody responses mounted against one or more antigen present on the E. coli offer a degree of protection. All EHECs appear to possess an R3 LPS core (Currie & Poxton, 1999; Amor et al., 2000).

The aim of this study was to test the hypothesis that cattle are exposed to R3 core LPS more frequently than humans, and consequently may develop an immune response against this LPS core type that could afford protection against EHEC infection.

**METHODS**

**Bacteria.** Bovine commensal isolates (n = 162) were kindly provided from the commensal E. coli collections of Dr Fiona Thomson-Carter of the University of Aberdeen and Dr Michael Pearce of the Wellcome Trust IPRAVE study. Isolates were representative of those from cattle from around Scotland. None of the isolates were O157 or verotoxin-producing E. coli (VTSEC). Human commensal E. coli isolates were obtained from 156 healthy volunteers aged approximately 20 years at the University of Edinburgh. Routine cultures were made on Columbia agar supplemented with horse blood (5 %) overnight at 37 °C. Strains were stored on nutrient agar slopes at 4 °C until required after inoculating single colonies and overnight incubation at 37 °C.

**Healthy human sera.** Sera were obtained from 91 healthy human volunteers at the University of Edinburgh. These were different from those volunteers who donated faecal samples. Following preliminary titrations of several samples by serum ELISA (see below), it was decided to use a dilution of 1 in 400 in PBS dilution buffer pH 7.4 (4 %, w/v polyethylene glycol 6000 and 0.05 %, v/v Tween 20) in ELISA procedures (see below). This dilution was selected as it was towards the top of the titration curve of all the sera tested, but well below the saturation point of the assay.

**Healthy bovine sera.** Dr Chris Low of the Scottish Agricultural College, Edinburgh provided the bovine test sera (n = 39) used in this study. Sera had been collected as part of the on-going screening of cattle for commercially important bovine diseases, and represented animals from all over Scotland. These sera were not from the same cattle from which the E. coli strains had been isolated. As for the human sera, preliminary titrations of several samples of serum were done. For the cattle sera it was decided to use a dilution of 1 in 1000 in PBS dilution buffer, pH 7.4 (4 %, w/v polyethylene glycol 6000 and 0.05 %, v/v Tween 20).

**PCR.** E. coli were subcultured on blood agar prior to lysis preparation. A single colony was emulsified in 100 μl sterile ultrapure water (Sigma). Subsequent heating at 100 °C for 10 min ensured complete lysis of all the bacteria. The resultant lysate formed the template from which all reactions proceeded. PCR was performed using published primer sequences (Amor et al., 2000). Multiplex PCR was not performed. Amplification of the target DNA was achieved by 35 cycles at 94 °C for 20 s, 50 °C for 30 s and 72 °C for 2 min 15 s. PCR products were resolved by 1 % agarose gel electrophoresis. Each gel was supplemented with 2 nM ethidium bromide to allow visualization of the DNA. Initially all isolates were screened for R1, then any failing to generate products were rescreened in turn for R2-, R3-, R4- and K12-specific genes until each had been assigned a core type.

**Determination of LPS core type by dot blot.** E. coli strains were subcultured overnight at 37 °C on Columbia agar supplemented with horse blood. Single colonies were suspended in 100 μl water. Nitrocellulose membranes were cut and marked into grids with squares of dimensions 10 × 10 mm. The nitrocellulose grids were washed in Tris buffered saline (TBS: 0.02 M Tris/HCl, 0.5 M NaCl, pH 7.5) and allowed to dry at 37 °C. Volumes (5 μl) of each bacterial suspension were applied to each of the squares on the nitrocellulose membrane. Controls comprising heat-killed cells and phenol/chloroform petroleum (PCP)-extracted core LPS representing core types R1, R2 and R3 were included on each nitrocellulose grid. Nitrocellulose was incubated at 37 °C to dry. Membranes were blocked with fish gelatin (3 %, v/v teleostean gelatin; Sigma) in PBS (pH 7.4; Oxoid) for 45 min at room temperature. Monoclonal antibodies as described by Gibb et al. (1992) were used for the determination of LPS core type. Monoclonal antibodies were diluted 1 in 50 in 1 % fish gelatin + PBS (pH 7.4) prior to use. Nitrocellulose membranes were incubated in 30 ml of the appropriate monoclonal antibody solution for 3 h at room temperature. Membranes were then washed twice in Tween-Tris buffered saline (TTBS: 0.02 M Tris/HCl, 0.5 M NaCl, 0.025 % Tween 20, pH 7.5) for 10 min. Rabbit anti-mouse IgG horseradish peroxidase (HRP; Sigma) was diluted 1 in 1000 in 1 % fish gelatin + PBS and incubated with the nitrocellulose membranes for 1 h 30 min. Membranes were washed twice in TTBS for 10 min, rinsed in H2O and developed in HRP colour reagent (Bio-Rad).

**LPS extraction by PCP and rapid aqueous phenol methods.** PCP-extracted LPS cores R1, R2, R3, R4 and K12 were prepared by the method of Galanos et al. (1969), as described by Hancock & Poxton (1988).

**Core-polymyxin conjugate.** This was by the method originally described by Scott & Barclay (1987). PCP-extracted LPS cores R1, R2, R3, R4 and K12 were prepared as 1 mg ml⁻¹ solutions with pyrogen-free water (pH2O). Each LPS solution was sonicated at 5 °C for 15 s to aid dispersal. Polymyxin-B sulphate was prepared as a 1 mg ml⁻¹ solution with pH2O. Volumes (1 ml) of the LPS solutions were added to an equal volume of the polymyxin-B sulphate solution. The resultant solution was sonicated as before. The polymyxin-B sulphate LPS core solution was then transferred to dialysis tubing (MWCO 2000; Spectrapore). The core-polymyxin solution was dialysed overnight at 4 °C with constant stirring, against 2 l pH2O to remove any unbound polymyxin-B sulphate.

**ELISA.** Polymyxin B sulphate–LPS core complexes were floccular upon recovery from the dialysis tubing. Complexes were transferred to glass bijoux and floccular masses were dispersed by sonication at 5 μm for 30 s. Coating buffer (0.05 M sodium carbonate buffer, pH 9.6) was used to dilute the complexes 1 in 50 prior to coating the plate. To coat, 100 μl of each complex was added to each well of a microtitre plate (Greiner bijoux and floccular masses were dispersed by sonication at 5 μm for 30 s. Coating buffer (0.05 M sodium carbonate buffer, pH 9.6) was used to dilute the complexes 1 in 50 prior to coating the plate. To coat, 100 μl of each complex was added to each well of a microtitre plate (Greiner medium binding strips; Labotechnik). The coating solution was vortexed at regular intervals to prevent large LPS-polymyxin complexes becoming coated on the ELISA plate. Coating was done overnight at 4 °C. Plates were washed four times with PBS-Tween (PBS, 2.6 mM KCl, 0.13 M NaCl, 0.05 % Tween 20, pH 7.4) and then blocked overnight at 4 °C with PBS + 3 % fish gelatin. Plates were then washed and dried and
stored at −20 °C until required. Volumes (100 μl) of human serum diluted 1 in 400 or bovine serum diluted 1 in 1000 in PBS-Tween were added in duplicate to wells of microtitre plates and incubated at 37 °C for 90 min. Dilution buffer only was applied to those wells used as negative controls. Antibody–antigen complexes were detected with the use of either mouse anti-human IgG HRP (Sigma) or sheep anti-bovine IgG HRP (Serotech), incubating at 37 °C for 90 min. HRP colour development was done with hydrogen peroxide and tetramethylbenzidine dihydrochloride (Sigma) in pH 5.0 buffer as recommended by the manufacturers, the results were read at 450 nm after incubation at room temperature for 20–30 min depending on colour change of a standard serum included on each plate which gave a colour near to the mean. All results were normalized to this standard.

Statistics. The statistical tests used were the Chi-squared test for the proportions of core types and Student’s t-test for the box plots.

RESULTS AND DISCUSSION

LPS core type detection by PCR and dot blot

Human faecal isolates obtained from 156 healthy human volunteers and 162 bovine isolates were screened for LPS core type by PCR. Lysates of each of the commensal isolates were prepared and used as the DNA template for PCR. Monoclonal antibodies were used to confirm the results from the PCR.

All human and bovine faecal E. coli isolates were screened for genes for the specific enzymes involved in the synthesis of each of the five known LPS core types (R1–R4 and K12) by the method of Amor et al. (2000). The primers designed to amplify the R1-specific region produce a product of 551 bp, while R2 E. coli produce a product of 1141 bp, R3 one of 1785 bp, R4 one of 699 bp and K12 one of 916 bp.

An example of the results of screening 35 isolates for the presence of genes specific to the synthesis of R3 core LPS by PCR is shown in Fig. 1(a). This shows that seven (lanes 8, 10, 19, 20, 24, 28 and 29) yielded a band at 1.7 kb indicative of R3 E. coli. The R3-specific monoclonal antibody was found to react with the same isolates and no others (Fig. 1b). The controls show two positive results where R3 LPS and heat-killed whole R3 E. coli were used. R1 and R2 core LPS was used for the negative controls; no reaction between the monoclonal antibody and these core types was observed. Similar specificity between both the R1 and R2 core-specific monoclonal antibodies and isolates found to be either R1 or R2 by PCR was observed. Antibodies specific to either the R4 or K12 core were not available.

The results for the distribution of LPS core types is summarized in Table 1. E. coli with the R1 core LPS was the most frequently isolated commensal from both healthy humans and cattle at 63 and 49 %, respectively. The numbers of R2 and R4 E. coli isolated from the healthy human faeces were very similar, 15 % being R2 and 14 % being R4. The number of R4 E. coli detected among bovine isolates was higher (25 %) than in humans, with R2 slightly lower. It was apparent that in humans R3 and K12 core LPS were the least common of all the core types, each being isolated in only 4 % of cases. Of note was the much higher level of R3 core LPS in the bovine commensals (11 %) as compared to the level in humans (4 %). This difference was statistically significant (P ≤ 0.05). It should be noted that in the initial screening experiments, several isolates did not produce a PCR product or react with monoclonal antibodies. These were subsequently discovered not to be E. coli (mainly Klebsiella spp.) and were discarded. All of the results including grand totals given in this paper do not include any of the strains which were not E. coli.
Table 1. The distribution of LPS core types within a population of faecal E. coli isolates from healthy humans and bovines

<table>
<thead>
<tr>
<th>Core type</th>
<th>No. (%) each core type within each faecal E. coli population</th>
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<tbody>
<tr>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>R1</td>
<td>98 (63)</td>
</tr>
<tr>
<td>R2</td>
<td>24 (15)</td>
</tr>
<tr>
<td>R3</td>
<td>6 (4)</td>
</tr>
<tr>
<td>R4</td>
<td>22 (14)</td>
</tr>
<tr>
<td>K12</td>
<td>6 (4)</td>
</tr>
<tr>
<td>Total</td>
<td>156</td>
</tr>
</tbody>
</table>

A previous study by Currie & Poxton (1999) showed that E. coli O157 and most other non-O157 VTECs carry the LPS core type R3. The study looked at VTECs, of which 20 were O157 and five were O111 with one each from serotypes O26, O128 and O86. All were found to carry R3 core LPS. More recently, studies by Amor et al. (2000) have confirmed that R3 core LPS is indeed often associated with VTEC. The study distinguished between VTECs and EHECs (verotoxin positive, but also carrying the LEE pathogenicity island and capable of producing human disease). They reported that all EHECs were R3-positive, but only 81% of the VTEC isolates tested were positive for R3 core LPS. These represented serotypes O26, O55, O91, O103 and O157, and several of the non-EHEC O157 isolates were found to have R1 core LPS. So, while there is not a complete association of R3 core LPS with the VTEC, it is by far the most common core type associated with the group.

Gibb et al. (1992) used core-specific monoclonal antibodies to determine which core types were the most prevalent among blood, urine and faecal isolates. Of all the isolates examined, the only true indication of the core type distribution among commensal E. coli can be obtained from the 21 faecal isolates examined. R1 core LPS was found to be most common (11 isolates), four isolates were found to carry R2 core LPS and two R3. Four were either R4 or K12 but were assigned no core type due to the lack of any anti-K12 or -R4 monoclonal antibodies. Appelmelk et al. (1994) investigated the distribution of LPS core types among 68 isolates from positive blood cultures. R1 core LPS was most frequently detected. Nine, 12 and seven strains were found to carry R2, R3 and R4 core LPS, respectively. Only three isolates were identified as being K12. Amor et al. (2000) examined 72 E. coli isolates from the E. coli reference collection (ECOR), deemed representative of the genetic diversity within the species, to obtain a view of the core type distribution in an unbiased population. R1 core LPS was found to be the most common core type. R2 and R3 core LPS was found in 11-1% of the isolates with R4 and K12 being found in only 2-8 and 5-6% of the isolates, respectively. However, the results presented by Appelmelk et al. (1994) and those presented by Amor et al. (2000), although representative of the whole E. coli species, may not accurately reflect the distribution of core types within commensal flora. Subtle relationships between LPS core type and the virulence determinants required to allow an organism to survive in a particular niche may affect the results.

While studies such as these have focused on the core types associated with a particular group of E. coli, for example the VTECs, the study undertaken here was intended to show how the LPS core types were distributed among a population of commensal E. coli from both healthy humans and cattle. The results would reveal which core type humans and cattle were most frequently exposed to in health and whether or not this had any influence on the level of the anti-core LPS antibody response.

Core LPS antibody responses in healthy humans and cattle

Currie et al. (2001) showed that patients convalescing from O157 infection had elevated levels of IgA to O157 LPS that could be attributed, in part, to the R3 core component. This implied that an immune response to core LPS may play a role in protection.

Serum from healthy humans and cattle was tested for the presence of antibodies reactive to each of the five E. coli LPS core types (R1–R4 and K12) as described in Methods. In all, 91 human (Fig. 2a) and 39 bovine serum samples were screened (Fig. 2b). It was found that in both cases the level of anti-R4 core antibody was statistically higher than the level of any other anti-core antibody (P < 0.01). This agrees with investigations in Germany on human sera (H. Brade, personal communication). In humans there was no statistical difference between the level of anti-R1, -R2 and -R3 core antibodies (P = 0.3 and 0.9). In both humans and cattle the level of anti-K12 antibody was significantly lower than the level of any other core type response (P < 0.01). As described earlier, R4 strains are much more common in faeces than K12 strains. This is reflected in the higher level of antibodies to the R4 core compared to K12. A possible reason for this is that the host immune system is more commonly exposed to R4 strains – by natural translocation from the GI tract. Moreover, R4 strains are apparently rarely found in systemic disease (Gibb et al. 1992). Is this because they are serum sensitive, or is it because antibody levels are higher?

Gibb et al. (1992) investigated the serum sensitivity of the isolates used in their study. They found that 81% of the R1 isolates were serum resistant, while 68% of other core types were found to be resistant (R4 and K12 core types not included). This was thought to be attributable to the capsule (K1 or K5) associated with the R1 isolates. The K1 and K5 capsules are poorly immunogenic due to the fact that they are composed of molecules mimicking those of the host (Devine & Roberts, 1994).

Devine & Roberts (1994) reported that the level of serum sensitivity of a particular isolate was most probably linked to both capsule type and O-type. A wide range of serum sensitivities was found among those isolates with K1 and
K5 capsules. Interestingly, serotypes O7, O18 and O75 (representing R1 and R2 core types) were found to be sensitive to killing by the alternative complement pathway but not by the classical pathway. This may suggest that exposure to the LPS core was blocked by a capsule, preventing effective antibody mediated-complement activation. Forat et al. (1992) showed that the serum sensitivity of various serotypes of *E. coli* depended upon the lengths of the O-antigen polysaccharide side chains. It is possible, therefore, that R4 *E. coli* are often without capsule or have O-antigens that do not provide serum resistance. This would correlate with the finding here that while the frequency of R4-carrying commensals is not significantly higher than the level of either R1 or R2 isolates, there is a significantly higher antibody response to the R4 core. This could result from the antigen challenge delivered by a serum-sensitive R4 organism when destroyed by both innate and acquired components of the immune system.

Of particular note was the elevated level of anti-R3 core antibody relative to the R1, R2 and K12 levels among the bovine sera tested. This was found to be statistically higher (*P* < 0.01) than the level of anti-R1 and -R2 core antibody within the same population.

A number of studies have examined the correlation between levels of anti-endotoxin core antibody (EndoCAb) and the development of postoperative complications. Bennett-Guerrero et al. (1997) postulated that endotoxin was one of the factors leading to pro-inflammatory responses in patients recovering from major heart surgery. Strutz et al. (1999) showed that low levels of anti-core LPS antibodies reduced the possibility of survival. In that study, however, serum was obtained upon admission to the intensive care unit, or upon diagnosis of sepsis, in which case the low antibody levels could have been attributed to a ‘mopping up’ effect of the antibody by the organism causing sepsis. Bennett-Guerrero et al. (1997) investigated links between postoperative complications and the level of IgG and IgM EndoCAb levels in 301 cardiac patients prior to surgery. They found that patients with low levels of antibody to the core were more likely to suffer complications.

In a comparative study between healthy volunteers from Edinburgh, UK and Dhaka, Bangladesh, Hoque et al. (2000) showed that there were significant differences in the levels of anti-core LPS antibodies between the two populations. Of note was the higher level of antibody to the R1, R3 and R4 core LPS in the population from Dhaka as compared to those in Edinburgh. It was proposed that this difference was because of high exposure of the Dhaka population to more gut pathogenic *E. coli* and shigellae (which have similar core types to *E. coli*) through their drinking water. It was also suggested that a role for both mucosal and systemic anti-core LPS antibodies was important in protection against GI and systemic *E. coli* and shigella infections. Could an increase in exposure to various *E. coli* O-serotypes lead to an increase in the level of antibody reactive to the core possessed by the specific serotypes?

Using a Chi-squared test it was possible to show that the level of R3 core LPS among the bovine commensals was significantly higher (*P* < 0.05) than the level found in the human commensals. This would suggest that cattle are exposed to more *E. coli* serotypes carrying R3 core LPS than humans. The association of R3 core LPS with VTEC as demonstrated by Gibb et al. (1992), Currie & Poxton (1999) and Amor et al. (2000) would imply that this increased R3 exposure would be due largely to a greater number of species related to VTECs, EHECs and enteropathogenic *E. coli* within the commensal populations of cattle. The level of anti-R3 core antibody detected in the bovine sera was significantly higher than the R1, R2 and K12 response. Whilst no direct comparisons between the human and bovine results are possible, this trend was not seen in the human samples tested. It is possible that cattle, being exposed to more R3 core LPS, generate a more significant response to this core type than humans, who are infrequently exposed.

Core LPS may be divided into inner and outer regions, the outer region displaying more variation than the inner (Amor...
et al., 2000). Barclay (1995) showed that incomplete (Rc) core LPS comprising the lipid A portion, three KDO residues, three heptose residues and a single glucose, was the minimum structure required to produce antibodies cross-reactive to a large number of core LPSs. The variation in the outer core is often limited to differences in only one or two sugar residues. For example, R1 core LPS differs from R4 core LPS in a single β(1 → 3) linked glucose residue [β(1 → 4) linked galactose at the same position in the R4 core] (Amor et al., 2000). This similarity between core types may result in a large number of cross-reactive antibodies. Hence the differences in antibody titre observed in this study (Fig. 2a, b) are possibly due to these often subtle differences between core types. In which case the high level of anti-R4 core antibody observed in both healthy humans and cattle, is due to the single sugar difference in the outer core.

In conclusion, the LPS core types of E. coli carried differ somewhat between humans and cattle, with the main difference being in R3 carriage. This is reflected in the different levels of systemic antibodies detected in healthy humans and cattle, with the R3 being significantly higher in cattle compared to humans. Whether or not this results in differences in resistance to disease has yet to be determined.

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REFERENCES


