Project Title: Distribution and expression of the 14 kDa fimbrial gene among *Salmonella* Enteritidis isolates and potential as diagnostic and epidemiological tools

Project Code: UA 2E

Principal Investigator and Organisation

Dr Connor J Thomas
Department of Microbiology and Immunology,
University of Adelaide, Adelaide, South Australia, 5005
Telephone: (08) 8303 5396
Fax: (08) 8303 4362
Email: connor.thomas@adelaide.edu.au

Start date: 1993 **Finish date:** 1994

Major Objectives

The major objectives of the study are:

1. To examine the distribution of the gene sequences encoding a 14 kDa fimbrial protein produced by *Salmonella enterica* serovar Enteritidis among Australian isolates of this pathogen
2. To confirm that the gene sequence for the 14 kDa fimbrial protein does not occur in isolates of other *Salmonella* serogroups
3. To examine the diagnostic potential of the 14 kDa protein and the gene sequence for *Salmonella* Enteritidis.

Background

The incidence of non-typhoid salmonellosis in humans has been steadily increasing (Cohen & Tauxe, 1986, *Science* **234**:964-969; Rodriguez *et al.,* 1990, *Epidemiol. Infect.* **105**:21-27). In particular, poultry have been implicated as a major reservoir of *Salmonella enterica* serovar Enteritidis phage type 4; the strain associated with major outbreaks of disease. Indeed there is a large amount of literature that relates to the incidence and significance of this bacterial pathogen in North America and the United Kingdom. In the United Kingdom, *S.* Enteritidis has become the predominant clinical isolate (Cooke, 1990, *Lancet* **336**:790-793). Nevertheless, only moderate numbers of *S.* Enteritidis have been referred to the *Salmonella* Reference Laboratory from Australian sources. For example, 104 isolates were confirmed in 1989, 99 isolates in 1990 and 76 isolations in 1991 (Australian Salmonella Reference Laboratory, Adelaide, South Australia, Annual Report). Sixty-six were isolated from humans in 1991 and were predominantly phage type 4 (18 isolates) or phage type 26 (26 isolates). The remaining 10 *S.* Enteritidis were cultured from a variety of sources including avian, reptile, sewage and food. None of the phage type 4 isolates obtained were known to be associated with poultry or poultry products.

In poultry, *S.* Enteritidis can cause enterocolitis. However, most attention has focussed on the ability of the pathogen to infect chicken oviducts and as a result contaminate the contents of intact eggs (Humphrey *et al.,* 1989, *Epidemiol. Infect*. **103**:415-423). Given the widespread use of egg and egg products in the food industry it is therefore not surprising that food-borne infections caused by *S.* Enteritidis have received such interest. Consequently, rapid methods that identify

contaminated foods are both desirable and necessary to locate the source and route of spread of the infection.

There are a number of important virulence determinants that contribute to the overall pathogenesis of animals by salmonellae. Fimbriae, for example have been implicated as important structures that facilitate the initial interaction between the pathogen and host. *Salmonella spp.* produce mannose sensitive, Type I fimbriae, which are morphologically similar to, but immunologically distinct from those produced by *E. coli* (Clegg & Gerlach, 1987, *J. Bacteriol.* **169**:934-938; Korhonen *et al.,* 1980, *J. Bacteriol.* **144**:800-805; Purcell *et al.,* 1987, *J. Bacteriol.* **169**:5831-5834). However the role of these structures in pathogenesis is not well understood, especially since *Salmonella spp.* produce non-haemagglutinating Type 2 fimbriae that exhibit Type I morphology. Evidently, these fimbriae are Type I structures which lack an adhesin. Furthermore, other *salmonellae* express Type 3 fimbriae that agglutinate tannin treated erythrocytes. Nevertheless, these antigens may be diagnostically useful, given that they do not in general share a wide spectrum of immunological cross-reactivity. This is supported by DNA homology studies which have shown that Type I fimbrial genes isolated from *E. coli* have little sequence homology to those of the salmonellas (Buchanan *et al.,* 1985, *J. Bacteriol.* **162**:799-803; Clegg *et al.,* 1985, *Infect. Immun.* **48**:275-279; Clegg *et al.,* 1985, *Infect. Immun.* **50**:338-340; Purcell *et al.,* 1987, *J. Bacteriol.* **169**:5831-5834).

The situation is no clearer for *S.* Enteritidis. Indeed work by Kay and co-workers (University of Victoria, British Columbia, Canada) has demonstrated that this pathogen produces at least three immunologically distinct types of fimbriae, one of which is biochemically and structurally similar to Type I fimbriae produced by *S. typhimurium* (Feutrier *et al.,* 1988, *J. Bacteriol.* **170**:4216-4222; Muller *et al.,* 1991, *J. Bacteriol.* **173**:4765-4772; Collinson *et al.,* 1991, *J. Bacteriol.* **173**:4773-4781).

Collinson *et al.*, 1991 (*J. Bacteriol.* **173**:4773-4781) showed that *S.* Enteritidis produces a novel thin aggregative type of fimbriae for which there is no known function. These fimbriae have a molecular weight of 17 kDa and are known as SEF17. The N-terminal amino acid sequence of these fimbriae shows no homology to any of some 40 fimbrial sequences and the distribution and immunological cross-reactivity of this antigen is unknown at this time (Figure 1). This fimbrin is expressed on solid and liquid media at both 30°C and 37°C.

S. Enteritidis also produces two other fimbriae which are similar morphologically, but quite biochemically (Figure 1) and antigenically distinct (Feutrier *et al.,* 1988, *J. Bacteriol.* **170**:4216-4222; Muller *et al.,* 1991, *J. Bacteriol.* **173**:4765-4772). Both are morphologically typical of Type I fimbriae, but only one is responsible for the mannose sensitive (MSHA) haemagglutination observed for this pathogen. The MSHA fimbrial sub-unit protein has a molecular weight of 21 kDa (SEF21), while the other morphologically related fimbrial sub-unit has a molecular weight of 14 kDa (SEF14). Both fimbrial types can be co-expressed under certain culture conditions, but SEF14 is only produced at temperatures above 30°C. SEF21 and SEF14 have unrelated N-terminal amino acid sequences.

Figure 1: Comparison of N-terminal sequences of fimbrin sub-unit proteins of aggregative and Type I fimbriae produced by *Salmonella*

Structure	Organism	Residue
SEF14	*S.* Enteritidis	AGFVGNKAVVQAAVTIAAQNTTSANWSQDPGFTGPA-
SEF21	*S.* Enteritidis	ADPTPVSVSGGTIHFEGKLVNAAA?VS??SAD-
TYPE I	*S.* Typhimurium	ADPTPVSVSGGTIHFEGKLVNAACAVSTKSAD-
SEF17	*S.* Enteritidis	GVVPQWGGGGNHN-
CFA/I	*E. coli* (ETEC)	VEKNITVTASVDPVIDL-
CFA/II	*E. coli* (ETEC)	AAGPTLTKELALNVLSP-
K88	*E. coli* (ETEC)	WMTGDFNGSVDIGGSIT-

Source: Adapted from Muller et al., 1991, J. Bacteriol. 173:4765-4772; and Collinson et al., 1991, J. Bacteriol. 173:4773-4781

Western immunoblot analyses by Kay and Trust's group, showed that immune sera raised against SEF21 are cross-reactive with fimbrial proteins produced by other *Salmonella spp.,* but do not react with Type I fimbriae produced by *E. coli.* This is consistent with data that show that the N-terminal sequence of *S.* Typhimurium is identical with SEF21 and that both fimbrins have similar amino acid composition (Figure 1). However, immune sera raised against SEF14 did not cross react with a panel of *Salmonella spp*. other than isolates of *S.* Enteritidis. This suggests that SEF14 are unique to *S.* Enteritidis strains. Support for this hypothesis is provided in part by Thorns *et al.*, 1990 (*J. Clin Microbiol.* **28**:2409-2414). These workers isolated a monoclonal antibody that reacted with epitopes associated with a 14.3 kDa fimbrial protein isolated from a strain of *S.* Enteritidis. This monoclonal reacted with all *S.* Enteritidis isolates tested, but also reacted with epitopes expressed by some strains of *S. dublin* and *S. moscow*. Representative isolates of 17 other unrelated *Salmonella* serogroups did not react with this antibody. Recent work by Ieva Kotlarski (Dept. Microbiology and Immunology, University of Adelaide) has resulted in isolation of a number of monoclonal antibodies that react with a 14 kDa protein expressed by *S.* Enteritidis strain 11RX. These monoclonals react with epitopes that are normally exposed on the native protein. N-terminal analysis has shown that this protein is likely to be the SEF14 fimbrial antigen described by Trust's group. The gene encoding this protein has been cloned and sequence analysis should confirm the identity of the gene product.

All of the available evidence that relates to characterisation of fimbrial antigens expressed by *S.* Enteritidis suggests that SEF14 may be unique to this pathogen. Consequently, it may be possible to capitalise on this observation and develop a novel strategy for rapid identification of *S.* Enteritidis in egg and egg products based on detection of DNA sequences encoding SEF14 or indeed by using an ELISA based assay, using antibodies raised against purified fimbrial protein. Colonies of Group D *Salmonella* such as *S.* Enteritidis may be rapidly screened using O9 polyvalent antisera. However, pre-incubation in media for up to 5 days may be required to obtain sufficient growth of the organism, and the test is not specific for *S.* Enteritidis. Immuno-assays for *S.* Enteritidis using lipopolysaccharide, outer membrane proteins and flagellar antigens have been described previously. For example, Kim *et al.*, 1991 (*Am. J. Vet. Res.* **52**:1069-1074) have shown that antibodies against outer membrane proteins provided the basis for an ELISA based test which is more sensitive than standard methods for detection. Lipopolysaccharide preparations from *S. gallinarum* and *S.* Enteritidis have also been employed in disc ELISA assays, but these approaches suffer from cross reactions with other *salmonellae* (Minga & Wray, 1992, *Res. Vet. Sci.* **52**:384-386; Nicholas & Cullen, 1991, *Vet. Rec.* **128**:74-76). It is evident from an examination of the literature that a need exists for a specific and sensitive method for detection of this potentially important pathogen.

Methods

Isolates used in this study

Isolates used in this study are listed in Table 1 and Table 2. Strains listed in Table 2 are part of an undescribed *S.* Enteriditis collection set maintained by the Department of Microbiology and Immunology, University of Adelaide. All strains are available as freeze dried cultures.

Table 1: List of *S.* Enteriditis strains used in this study, specificity of polymerase chain reaction for detection of a region of *sefA* and *sefABC* and presence of *SefA* antigen detected by slot immunoblot analysis

Strain	Identity	Source	Origin	Presence of PCR Amplicon		SefA
				526 bp	3657 bp	
93/04153	*S.* Enteritidis	chicken	Singapore	+	+	+
93/04438	*S.* Enteritidis	human	S.A	+	nd	+
93/4439	*S.* Enteritidis	human	S.A	+	+	+
93/03247	*S.* Enteritidis	human o/s	S.A	+	+	+
93/03290	*S.* Enteritidis	human	S.A	+	+	+
93/03565	*S.* Enteritidis	unknown	N.S.W	+	nd	+
93/02488	*S.* Enteritidis	human o/s	Q.L.D	+	+	+
93/02352	*S.* Enteritidis	human o/s	Q.L.D	+	nd	+
93/01410	*S.* Enteritidis	chicken	Singapore	+	+	+
93/01408	*S.* Enteritidis	chicken	Singapore	+	nd	+
93/00811	*S.* Enteritidis	human	Q.L.D	+	nd	+
93/01107	*S.* Enteritidis	human	Q.L.D	+	nd	+
93/00874	*S.* Enteritidis	human	N.T	+	nd	+
93/00770	*S.* Enteritidis	human o/s	N.S.W	+	nd	+
93/00557	*S.* Enteritidis	human o/s	Q.L.D	+	nd	+
93/00034	*S.* Enteritidis	human	S.A	+	+	+
92/08173	*S.* Enteritidis	human	N.S.W	+	+	+
92/08288	*S.* Enteritidis	human	Q.L.D	+	nd	+
92/08504	*S.* Enteritidis	human	Q.L.D	+	nd	+
92/08319	*S.* Enteritidis	human	S/A	+	nd	+
92/08622	*S.* Enteritidis	human o/s	Q.L.D	+	nd	+
92/08962	*S.* Enteritidis	human o/s	Q.L.D	+	+	+
92/07904	*S.* Enteritidis	human o/s	Q.L.D	+	nd	+
92/07791	*S.* Enteritidis	human o/s	S.A	+	nd	+
7292	*S.* Enteritidis		UofAd	+	+	+
M386	*S.* Stanley		UofAd	-	-	-
D9133	*S.* Typhimurium		UofAd	-	-	-
M375	*S.* Cholerae suis		UofAd	-	-	-
C5(62)	*S.* Typhimurium		UofAd	-	-	-
11RX	*S.* Enteritidis		UofAd	nd	+	+
7314	*S.* Enteritidis		UofAd	+	+	+
	Serratia marcesens		UofAd	-	-	nd
	Escherichia coli DH1		UofAd	-	-	nd
	Xenorhabdus bovienii		UofAd	nd	nd	nd

KEY: + amplicon detected; - amplicon not detected; nd not done; UofAd, University of Adelaide, Department of Microbiology and Immunology collection.

Table 2: List of *S.* Enteriditis strains used in this study, specificity of polymerase chain reaction for detection of a region of *sefA* and presence of *sefA* antigen detected by slot immunoblot analysis

Strain	Identity	Source	Presence of PCR Amplicon		SefA
			526 bp	3657 bp	
621532	*S.* Enteriditis	UofA	+	nd	+
621533	*S.* Enteriditis	UofA	+	nd	+

Strain	Identity	Source	Presence of PCR Amplicon		SefA
622099	*S.* Enteriditis	UofA	+	nd	+
622204	*S.* Enteriditis	UofA	+	nd	+
622204	*S.* Enteriditis	UofA	+	nd	+
623035	*S.* Enteriditis	UofA	+	nd	+
623037	*S.* Enteriditis	UofA	+	nd	+
623046	*S.* Enteriditis	UofA	+	nd	+
623047	*S.* Enteriditis	UofA	+	nd	+
624031	*S.* Enteriditis	UofA	+	nd	+
624032	*S.* Enteriditis	UofA	+	nd	+
627706	*S.* Enteriditis	UofA	+	nd	+
627708	*S.* Enteriditis	UofA	+	nd	+
627709	*S.* Enteriditis	UofA	+	nd	+
627711	*S.* Enteriditis	UofA	+	nd	+
628278	*S.* Enteriditis	UofA	+	nd	+
630041	*S.* Enteriditis	UofA	+	nd	+
630047	*S.* Enteriditis	UofA	+	nd	+
630048	*S.* Enteriditis	UofA	+	nd	+
630615	*S.* Enteriditis	UofA	+	nd	+
631395	*S.* Enteriditis	UofA	+	nd	+
631571	*S.* Enteriditis	UofA	+	nd	+
631571	*S.* Enteriditis	UofA	+	nd	+
632499	*S.* Enteriditis	UofA	+	nd	+
633416	*S.* Enteriditis	UofA	+	nd	+
633913	*S.* Enteriditis	UofA	+	nd	+
634714	*S.* Enteriditis	UofA	+	nd	+

KEY: + amplicon detected; - amplicon not detected; nd not done; UofAd, University of Adelaide, Department of Microbiology and Immunology collection.

Polymerase Chain Reaction (PCR)

The presence and distribution of gene sequences encoding the SEF14 fimbrial gene was determined by Polymerase Chain Reaction (PCR) amplification of a 526 bp DNA fragment internal to *sefA* using oligonucleotide primers identified from available Genbank sequence data (Accession numbers L03833 and L11008). The primers used were: #808 5'-tgtgcgaatgctaatagttg-3', and #809 5'-ctgctgaacgtagaaggtcg-3'. A schematic identifying the approximate positions and orientation of these primers with respect to the *sefABC* sequence data is shown in Figure 2 and Figure 3. Amplification was carried out in a thermocycler as follows: denaturation at 94°C followed by 30 cycles of incubation at 55°C for 30 seconds, 72°C for 60 seconds, 94°C for 30 seconds followed by a final incubation step at 72°C for 5 minutes.

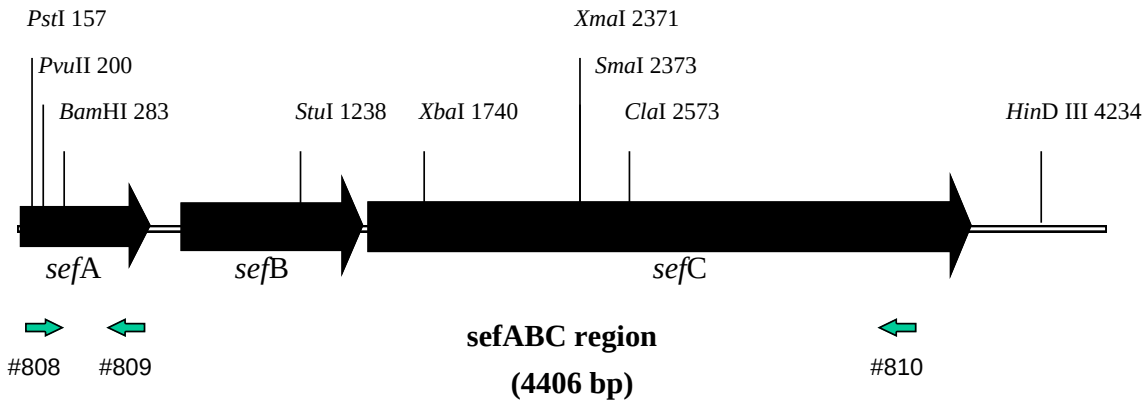


Figure 2: Diagramatic representation showing the genetic organisation of the *sef*ABC operon (approximately to scale), location of some common restriction endonuclease recognition sites (sequence position shown; approximately to scale) and the location and orientation of the oligonucleotide primers (#808, #809 and #810) (not to scale) used in this study.

Figure 3: Complete Genbank sequence entry encompassing the *sefABC* region. The position of the oligonucleotide primers #808, #809 (reverse) and #810 (reverse) are underlined.

```
LOCUS       I44902       4406 bp    DNA             PAT       07-OCT-1997
DEFINITION  Sequence 39 from patent US 5635617.
ACCESSION   I44902
VERSION     I44902.1  GI:2469615
KEYWORDS    .
SOURCE      Unknown.
ORGANISM  Unknown.
          Unclassified.
REFERENCE   1  (bases 1 to 4406)
  AUTHORS   Doran,J.L., Kay,W.W., Collinson, S.Karen and Clouthier,S.C.
  TITLE     Methods and compositions comprising the agfA gene for detection of
          Salmonella
  JOURNAL   Patent: US 5635617-A 39 03-JUN-1997;
FEATURES             Location/Qualifiers
     source          1..4406
                     /organism="unknown"
BASE COUNT     1483 a    675 c    852 g   1396 t
ORIGIN
        1 ggggatgttg tgtaaagata aaaaaatagt gatccttgtt ttttttctta aatttttaaa
       61 atggcgtgag tatattagca tccgcacaga taaat[tgtgc gaatgctaat agttg]atttt
                                          Primer #808
      121 tggagatttt gtaatatgcg taaatcagca tctgcagtag cagttcttgc tttaattgca
      181 tgtggcagtg cccacgcagc tggctttgtt ggtaacaaag cagtggttca ggcagcggtt
      241 actattgcag ctcagaatac aacatcagcc aactggagtc aggatcctgg ctttacaggg
      301 cctgctgttg ctgctggtca gaaagttggt actctcagca ttactgctac tggtccacat
      361 aactcagtat ctattgcagg taaaggggct tcggtatctg gtggtgtagc cactgtcccg
      421 ttcgttgatg gacaaggaca gcctgttttc cgtgggcgta ttcagggagc caatattaat
      481 gaccaagcaa atactggaat tgacgggctt gcaggttggc gagttgccag ctctcaagaa
      541 acgctaaatg tccctgtcac aacctttggt aaatcgaccc tgccagcagg tactttcact
      601 g[cgaccttct acgttcagca g]tatcaaaac taatttaatt taaactttat aaatgccctc
             Primer #809
      661 aatatgaggg catttggata attttattat tttaaaaata tctattttga atagataggt
      721 tttatgcttc catgcaaaaa cttaaagagg gattatgtat attttgaata aatttatacg
      781 tagaactgtt atctttttct ttttttgcta ccttccaatt gcttcttcgg aaagtaaaaa
      841 aattgagcaa ccattattaa cacaaaaata ttatggccta agattgggca ctacacgtgt
      901 tatttataaa gaagatgctc catcaacaag tttttggatt atgaatgaaa aagaatatcc
      961 aatccttgtt caaactcaag tatataatga tgataaatca tcaaaagctc catttattgt
     1021 aacaccacct attttgaaag ttgaaagtaa tgcgcgaaca agattgaagg taataccaac
     1081 aagtaatcta ttcaataaaa atgaggagtc tttgtattgg ttgtgtgtaa aaggagtccc
     1141 accactaaat gataatgaaa gcaataataa aaacaacata actacgaatc ttaatgtgaa
     1201 tgtggttacg aatagttgta ttaaattaat ttataggcct aaaactatag acttaacgac
     1261 aatggagatt gcagataaat taaagttaga gagaaaagga aatagtatag ttataaagaa
     1321 tccaacatca tcatatgtga atattgcaaa tattaaatct ggtaatttaa gttttaatat
     1381 tccaaatgga tatattgagc catttggata tgctcaatta cctggtggag tacatagtaa
     1441 aataactttg actattttgg atgataacgg cgctgaaatt ataagagatt attagtttaa
     1501 ggtgtaaaca aatgaagaaa accacaatta ctctatttgt tttaaccagt gtatttcact
     1561 ctggaaatgt tttctccaga caatataatt tcgactatgg aagtttgagt cttcctcccg
     1621 gtgagaatgc atcttttcta agtgttgaaa cgcttcctgg taattatgtt gttgatgtat
     1681 atttgaataa tcagttaaaa gaaactactg agttgtattt caaatcaatg actcagactc
     1741 tagaaccatg cttaacaaaa gaaaaactta taaagtatgg gatcgccatc caggagcttc
     1801 atgggttgca gtttgataat gaacaatgcg ttctcttaga gcattctcct cttaaatata
     1861 cttataacgc ggctaaccaa agtttgcttt taaatgcacc atctaaaatt ctatctccaa
     1921 tagacagtga aattgctgat gaaaatatct gggatgatgg cattaacgct tttcttttaa
     1981 attacagagc taattatttg cattctaagg ttggaggaga agattcatac tttggtcaaa
     2041 ttcaacctgg ttttaatttt ggtccctggc ggctaaggaa tctatcatct tggcaaaact
     2101 tgtcaagcga aaaaaaattt gaatcagcat atatttatgc tgagcgaggt ttaaaaaaaa
     2161 taaagagcaa actaacagtt ggggacaaat ataccagtgc agatttattc gatagcgtac
     2221 catttagagg cttttcttta aataaagatg aaagtatgat acctttctca cagagaacat
     2281 attatccaac aatacgtggt attgcgaaaa ccaatgcgac tgtagaagta agacaaaatg
     2341 gatacttgat atattctact tcagtccccc ccgggcaatt cgagataggt agagaacaaa
     2401 ttgctgatct tggtgttggg gttggggttc ttgatgttag catttatgaa aaaaatgggc
     2461 aggtccaaaa ctatacagtg ccatattcaa ctcctgtatt atctttgcct gatggatatt
     2521 ctaaatatag tgtaactatt ggtagataca gggaggttaa caatgattat atcgatcctg
     2581 tttttttga agggacttat atatatggtc tgccttatgg gtttacttta tttggtggag
     2641 tgcaatgggt aaatatttat aattcatatg ccataggcgc aagtaaagat attggtgagt
     2701 atggtgctct gtcttttgac tggaaaacat ctgtttcgaa gactgataca tccaatgaaa
     2761 atggtcatgc atatgggatt agatacaata aaaatatcgc tcagacaaac accgaagtat
     2821 ctttggctag tcattactat tattcgaaaa attatagaac tttttctgaa gcaattcata
     2881 gtagcgagca tgatgaattt tacgataaaa ataagaaatc aacaacctct atgttattaa
     2941 gtcaggcatt aggatctctg ggttctgtta acttaagcta caattatgat aaatattgga
     3001 aacatgaagg taaaaaatca ataattgcta gttatgggaa gaatttaaat ggtgtttcgt
```

3061 tatcgctttc atatacgaaa agtacatcaa agattagtga agaaaatgaa gatttattca
3121 gttttctact cagtgtacct ttgcaaaaac ttacaaatca tgaaatgtat gctacatatc
3181 aaaactcatc ctcttcaaag catgatatga atcatgattt aggtattact ggtgtggcat
3241 ttaatagcca attgacatgg caagcaagag ggcaaataga agataaatcg aaaaatcaaa
3301 aggctacatt tttaaatgct tcttggcgag gtacttatgg ggagatcgga gcaaactata
3361 gtcataatga aataaatcgt gatattggga tgaatgtttc tggtggggtg attgctcatt
3421 catcaggaat tacgtttggt catagtatat cggatactgc tgcactggta gaggctaaag
3481 gtgtaagtgg ggcaaaagtt ctgggcctac caggtgttat aaccgatttt agaggctata
3541 caatatccag ttatcttact ccatatatga ataacttcat atctatagat ccaacaacgt
3601 taccaataaa tacggatatt aggcaaactg atattcaagt agttcctacc gaaggtgcta
3661 ttgtaaaagc tgtatataaa acaagcgtgg gtactaatgc attaattaga attacaagaa
3721 caaatggaaa gc**cactagct cttggcacag tt**ctttcact taagaataat gatggagtaa
Primer #810
3781 tccaatcaac atctattgtt ggcgaagatg gtcaggcata tgtatctgga ttgtcaggag
3841 tgcaaaaatt aatcgcttcg tgggggaata atccctccga tacttgtaca gttttttact
3901 ctcttcccga taaaaacaaa ggtcagatta gctttttaaa tggagtgtgc aaatgaatga
3961 atcagtataa ttcgtcaata cctaagttca ttgtctctgt ttttctgatt gttactggtt
4021 ttttcagctc aactattaaa gcacaagaac ttaaattaat gattaaaata aatgaggctg
4081 ttttttatga ccgtattaca agtaataaaa taataggtac ggggcatcta tttaacagag
4141 agggaaaaaa aatcctcatt agttcaagtt tagaaaaaat taaaaatacc ccaggggcat
4201 atattattag aggtcagaat aactcagccc ataagcttag gataagaata ggtggagaag
4261 actggcaacc agataattca ggtattggta tggtatctca ttctgatttt actaatgaat
4321 ttaatattta ttttttggg aatggagaca ttcctgttga cacatattta ataagcatat
4381 atgcgacaga aattgaatta taataa
//

The amplified product was confirmed by digestion with the restriction endonuclease enzyme, *Bam*H1. This enzyme cleaves the product to yield two fragments of 186 bp and 340 bp.

PCR was also used for attempts to identify restriction fragment length polymorphisms within contiguous DNA encoding *sefABC* (Accession numbers L11008, L11009, L11010). The following primer pair was used to amplify a 3657 bp fragment: #808 5'-tgtgcgaatgctaatagttg-3' and #810 5'-aactgtgccaagagctagtg-3'. The approximate positions and orientation of these primers with respect to the *sefABC* sequence data is shown in Figure 2 and Figure 3. Amplified product was digested with *Bam*H1, *Kpn*I, *Sac*I, *Nco*I, *Dra*I or *Hpa*I. Digested amplification products were separated on agarose gels by electrophoresis and fragment profiles for different isolates compared.

Southern Analysis

Restriction endonuclease enzyme digested DNA was subjected to Southern hybridisation analysis using standard techniques. DNA probes were prepared by PCR mediated incorporation of digoxigenin labelled-11-dUTP into the PCR products described above. Presence of digoxigenin labelled DNA on Southern blots was detected using anti-digoxigenin-conjugate technologies.

Isolation of Fimbriae

Crude preparations of fimbrial protein were prepared as follows. Bacterial strains were cultured overnight at 37°C on Nutrient agar. Cells were harvested by suspension of growth in 1 ml of phosphate buffered saline. The suspension was transferred to a plastic reaction tube and heat shocked at 56°C for 20 minutes in a water bath. Cells were pelleted by centrifugation in a microfuge. The supernatant containing fimbriae was collected and transferred to a fresh reaction tube. Samples of each preparation were used for SDS PAGE and Western analysis.

Western transfer and analysis

Expression of SEF14 fimbriae by *S.* Enteritidis cultures was examined using standard immunoblot procedures. Crude fimbrial preparations were prepared as described above. Presence of *sefA* encoded fimbrial protein was detected using a mouse monoclonal antibody. This antibody had been previously demonstrated to be specific for SEF14 fimbria expressed by a strain of *S.* Enteriditis strain 11RX. The monoclonal antibody was a gift from Professor I Kotlarski, Department of Microbiology and Immunology, University of Adelaide.

SDS PAGE analysis

Standard protocols were used for this procedure.

Results

Presence of sef *homologs in strains tested*

Primers #808 and #809 were successfully used to amplify a 526 bp amplicon from DNA prepared from each of the *S.* Enteriditis strains tested (26/26) (Table 1 and Table 2). Figure 4 shows typical results obtained for *S.* Enteriditis strains. DNA was not amplified from a variety of non-*S.* Enteritidis strains. The amplified products were confirmed by digestion with the restriction endonuclease enzyme, *Bam*H1. This enzyme cleaves the product to yield two fragments of 186 bp and 340 bp. When digested with *Bam*HI, each 526 bp PCR product amplified from DNA from all of the *S.* Enteritidis strains gave the predicted digestion pattern. Figure 5 shows typical results for PCR amplified DNA digested with *Bam*HI.

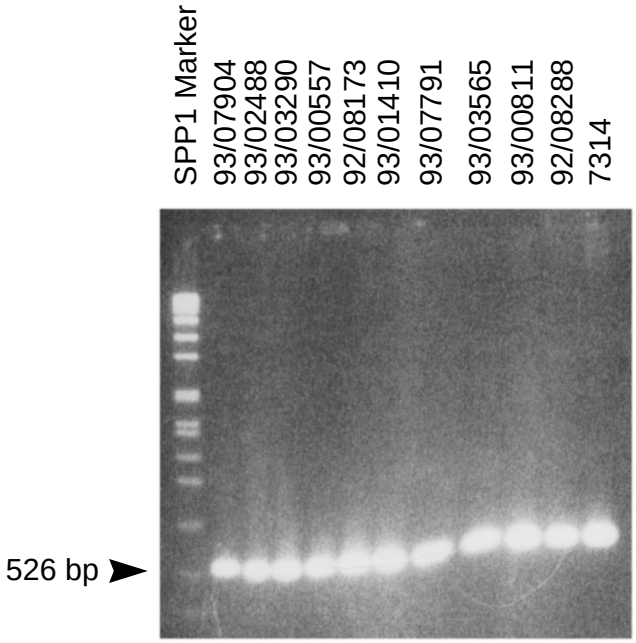


Figure 4: Agarose gel electrophoresis of PCR product amplified DNA isolated from *S.* Enteriditis strains. The 526 bp product was amplified using primers #808 and #809. Strain identity is as described in Table 1.

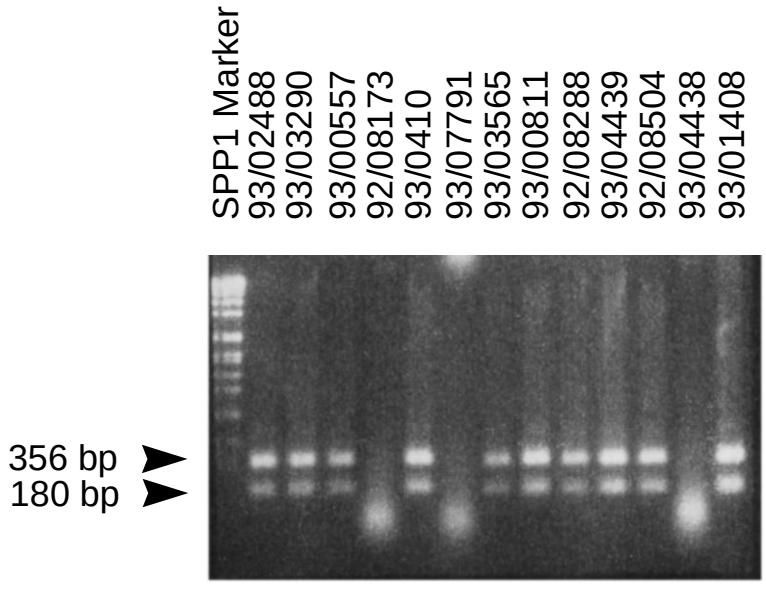


Figure 5: Agarose gel electrophoresis of BamHI digested PCR product amplified DNA isolated from *S.* Enteriditis strains. The 526 bp product was amplified using primers #808 and #809 then digested with BamHI. Note the 356 and 180 bp fragments typical of the sefA amplicon. Strain identity is as described in Table 1.

When primers #808 and #810 were used to amplify a 3657 bp fragment from the *sefABC* region of *S.* Enteritidis strains, this product was amplified from 9 of 9 randomly selected samples of DNA prepared from *S.* Enteriditis strains. A PCR product was not obtained from DNA isolated from six non-*S.* Enteriditis strains tested using the same primers.

Preliminary experiments were conducted to determine whether significant restriction fragment length polymorphisms existed within the *sefABC* region. Sequence data was first screened for restriction enzymes recognition sites for enzymes that cut in at least 5 places. Of several enzymes tested (*Bam*H1, *Kpn*I, *Sac*I, *Nco*I, *Dra*I or *Hpa*I), *Dra*I was the only useful enzyme that potentially digested the 3657 bp fragment into at least 5 fragments (predicted fragment sizes: 52, 87, 174, 267, 439, 545, 807 and 1198 bp). Digestion of the PCR products with *DraI* produced identical restriction patterns for all the 3657 bp fragments amplified from *S.* Enteriditis strains (Data not shown). Essentially identical results were obtained when the 3657 bp fragment from *S.* Enteriditis strain 11RX was used as a DNA probe in Southern hybridisation analysis of *Dra*I digested DNA isolated from *S.* Enteriditis strains (Data not shown). This experimental data suggested that *Dra*I restriction fragment length polymorphisms are not present in the 3657 bp region tested.

Expression of SefA *pilus protein by strains tested*

An anti-*SefA* monoclonal antibody (KAP16-1) was tested for ability to detect *SefA* expressing strains of *S.* Enteriditis. Preliminary experiments were conducted using crude pili preparations isolated from *S.* Enteriditis strains 11RX and 7314. The preparations were separated on SDS PAGE and the proteins blotted onto nitrocellulose membrane. The immunoblot was then probed with monoclonal antibody KAP16-1. Both strains expressed a 14 kDa protein that reacted with this monoclonal antibody preparation. Pili expression was maximal when strains were grown on either nutrient agar (NA) or colonisation factor agar (CFA) at 37°C (Figure 6). Only poor expression of the 14 kDa pilus protein was noted when these strains were grown at 28°C or in broth culture at either temperature. Subsequently, monoclonal KAP16-1 was shown to bind in colony immunoblot experiments. This monoclonal did not react with other non-*S.* Enteriditis test strains of bacteria (Figure 7).

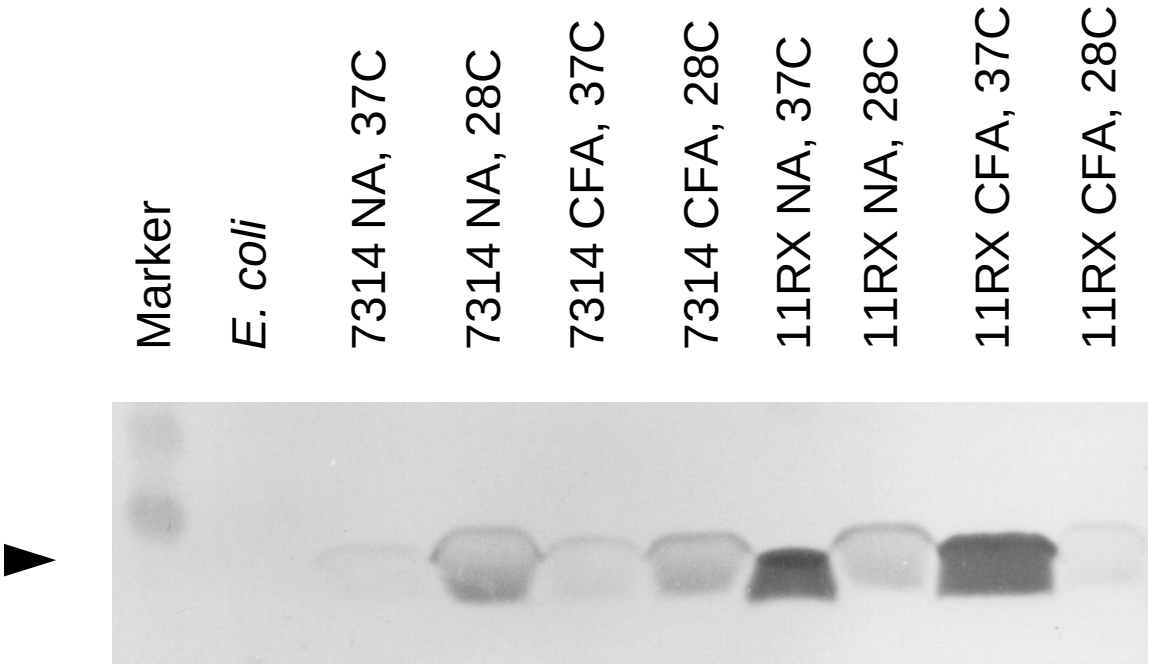


Figure 6: Effect of growth temperature and growth medium on expression of *SefA* pilin by *S.* Enteriditis strains 11RX and 7314. Strains were grown on Nutrient agar (NA) or Colonisation factor agar (CFA) at 28 or 37°C. Crude pili preparations were then run on SDS PAGE, and subjected to Western Immunoblot analysis using the KAP16-1 monoclonal antibody as a probe. The position of the 14kDA *SefA* pilin is arrowed.

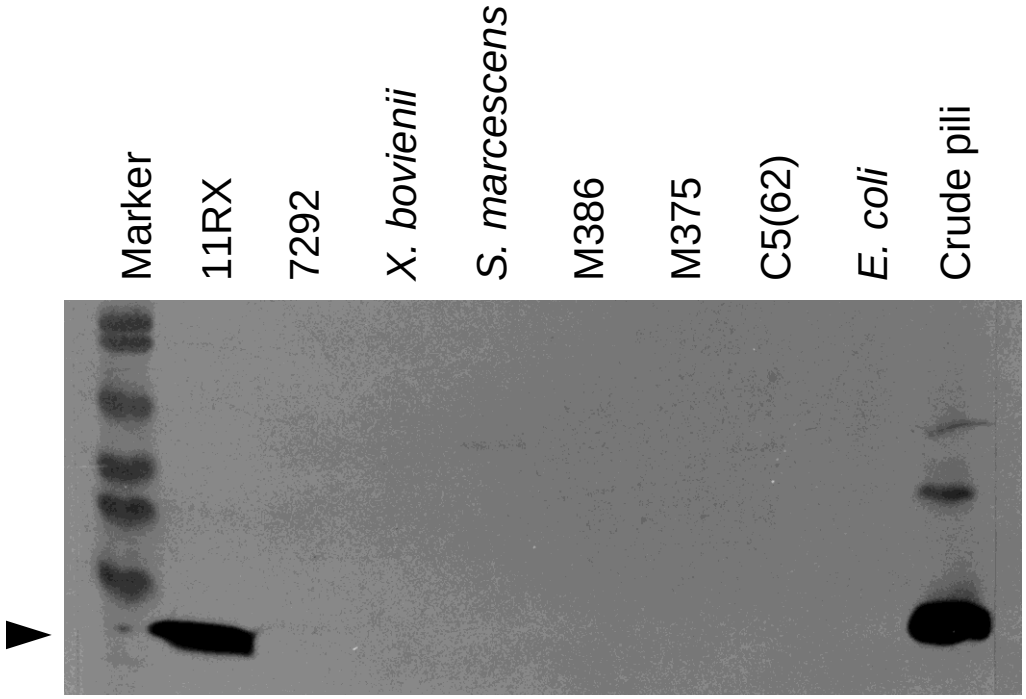
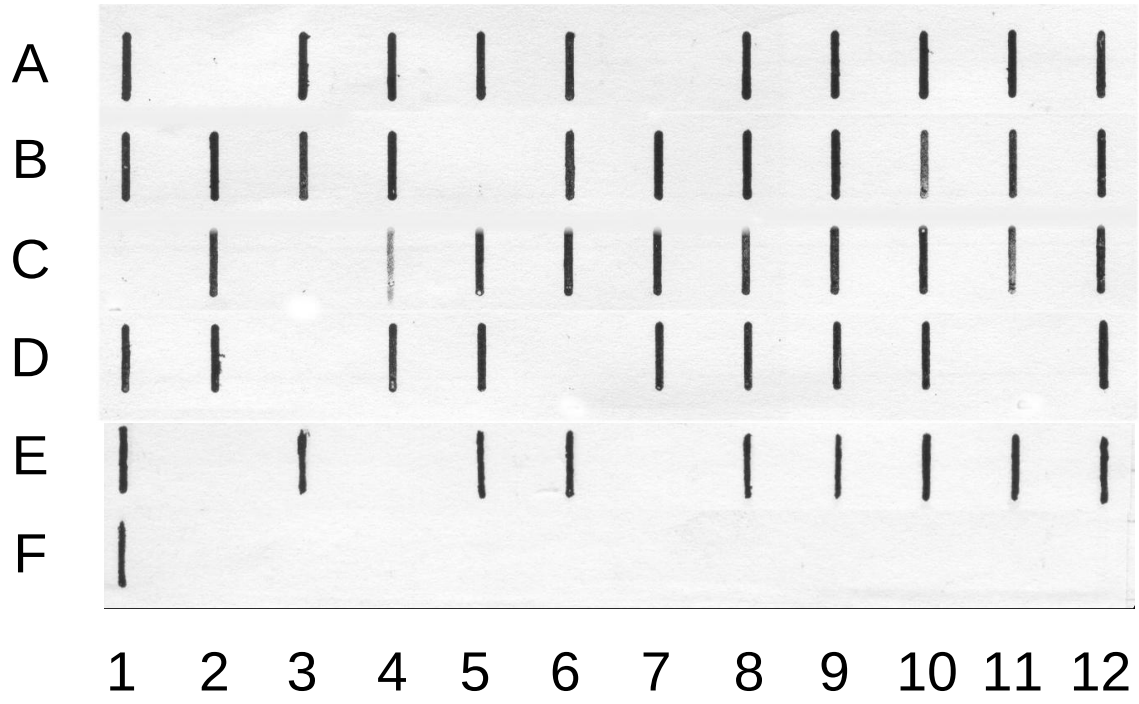


Figure 7: Effect of growth temperature and growth medium on expression of *SefA* pilin by *S.* Enteriditis strains 11RX other non-*S.* Enteriditis test strains. Strains were grown on Nutrient agar (NA) or Colonisation factor agar (CFA) at 28 or 37°C. Crude pili preparations were then run on SDS PAGE, and subjected to Western Immunoblot analysis using the KAP16-1 monoclonal antibody as a probe. The position of the 14kDA *SefA* pilin is arrowed. Strain identity is as indicated in Table 1.

To test the ability of this monoclonal to identify *S.* Enteriditis, test strains were grown on nutrient agar at 37°C overnight and colonies lifted onto nitrocellulose. The blots were treated with 0.5M HCl for 30 minutes then washed with saline to remove unbound cellular material. The blots were then incubated in blocking solution and reacted with appropriately diluted KAP16-1 monoclonal antibody. Variable results were obtained. The result obtained depended on the amount of cellular debris left on the nitrocellulose membrane. To overcome the problem of uneven distribution of cells on the membranes, test strains were grown in nutrient broth at 37°C and 200 □l of culture added to separate wells of a slot blot apparatus and the cells captured by suction onto the surface of nitrocellulose membrane. The membrane was treated with 0.5 M HCl for 45 minutes, rinsed in blocking solution and probed with monoclonal antibody. This method of preparation allowed routine detection of *SefA* from all *S.* Enteriditis strains tested. An example slot blot is shown in Figure 8. Although several *S.* Enteriditis strains were not detected in this blot (7292, 621533, 93/04153 and 622099) these exceptions were related to culture loading problems and subsequent repeats of the this work indicated these four strains reacted with KAP16-1 monoclonal antibody preparations. All 53 test strains of *S.* Enteriditis tested positive. None of the non-*S.* Enteriditis strains reacted with the monoclonal antibody when the slot blot method was employed.



Row/Column assignments:

Column	Row A	Row B	Row C	Row D	Row E	Row F
1	11RX	93/0057	M386	624031	11RX	93/03247
2	*E. coli*	92/08319	93/02352	621532	E. coli	
3	632499	627711	C5(62)	D9133	622204	
4	93/00811	631571	93/00874	624032	622099	
5	93/01408	M375	93/04438	627706	92/08962	
6	634714	628278	92/07791	621533	623037	
7	7292	623047	7314	627708	M386	
8	93/02488	93/03565	633913	93/01107	93/03290	
9	92/07904	631571	623046	623035	630041	
10	92/08504	630048	630047	627709	92/08288	
11	633416	93/00034	631395	93/04153	93/00770	
12	92/08622	622204	630615	92/08173	93/01410	

Figure 8: Expression of *SefA* antigen by test strains using a slot immunoblot analysis method. Monoclonal KAP16-1 was used as a probe. Test strain assignments to rows/columns are shown below. Strain identities are as indicated in Tables 1 and 2.

Conclusions

1. Primers #808 and #809 can be used to specifically amplify a 526 bp fragment of DNA from *S.* Enteriditis strains. These primers will not amplify a 526 bp fragment from DNA isolated from other *Salmonella* or non-*Salmonella* strains.
2. The presence of a conserved *Bam*HI restriction endonuclease recognition site within the 526 bp product can be used to confirm the identity of the product. Digestion of the PCR fragment produces two characteristic fragments with sizes of 340 bp and 186 bp.
3. In an attempt to determine whether the restriction fragment length polymorphisms existed within the *sefABC* region, PCR amplified DNA encompassing this region was amplified and digested with *Dra*I. A 3657 bp region internal to *sefABC* was amplified using primers #808 and #810 and digested with *Dra*I. Identical restriction digestion patterns were obtained for all strains tested. This result was confirmed by Southern hybridisation analysis of *Dra*I digested chromosomal DNA probed with labelled 3657 bp PCR amplified DNA. This suggested that *S.* Enteriditis strains do not contain epidemiologically useful *Dra*I restriction fragment length polymorphisms within the *sefABC* region. Computer analysis of existing DNA sequence data for the *sefABC* region did not identify other restriction endonucleases useful for restriction fragment length polymorphism analysis.
4. A monoclonal antibody preparation (SEF16-1) specific for the *S.* Enteriditis SefA pilus structural protein was assessed for potential as the basis for an immunological method for detection of *S.* Enteriditis strains. This antibody preparation was shown by Western Immunoblot analysis to be specific for semi-purified *S.* Enteriditis SefA pilus protein preparations. Furthermore, a slot immunoblot method was developed as a method for reliably detecting *S.* Enteriditis strains based on presence of the SefA pilus antigen.

Opportunities Arising

The results of this small study indicate that opportunity exists to use either PCR or antibody based techniques directed at the *sefA* gene or the SefA pilin sub-unit protein as techniques for rapid detection of *S. enterica* sv Enteriditis strains. A section of the *sefA* gene can be reliably amplified from *S.* Enteriditis strains and this amplicon can be easily confirmed by simple restriction endonuclease analysis. The primers identified could be incorporated into any of the new PCR detection based technologies currently available. However, the basic technique needs to be evaluated to determine base sensitivity and to determine whether the method needs to be applied to enriched cultures or whether it can be used directly with DNA extracted from raw materials. For example, there may be PCR inhibitors in egg white/yolk that prevent the use of direct PCR methodology on this raw product.

Similarly, this study has shown that monoclonal antibodies directed at the SefA protein could also be integrated into current immunological technologies as a rapid detection method either as the basis of direct immunofluorescent microscopy on raw or enriched culture material, or as the basis for ELISA based methods.

It should be noted that Doran *et al.* have issued a patent on methods and compositions comprising the sequence data used to develop the PCR based techniques used in this study. This patent (US 5635617-A 39 03-Jun-1997) was however lodged in 1997, some 5 years after the sequence data was placed on the Public Domain Genbank Database. Whether this patent affects use of the basic technologies described in this report remains to be assessed.