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# Application of Fluorescent Amplified Fragment Length Polymorphism for Comparison of Human and Animal Isolates of *Yersinia enterocolitica*

Catherine Fearnley,[1] Stephen L. W. On,[2] Branko Kokotovic,[2] Georgina Manning,[1] Tom Cheasty,[3] and Diane G. Newell[1]*

*Department of Food and Environmental Safety, Veterinary Laboratories Agency, New Haw, Surrey,[1] and Health Protection Agency, Colindale, London,[3] United Kingdom, and Danish Institute for Food and Veterinary Research, Copenhagen, Denmark[2]*

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**An amplified fragment length polymorphism (AFLP) method, developed to genotype *Yersinia enterocolitica*, has been used to investigate 70 representative strains isolated from humans, pigs, sheep, and cattle in the United Kingdom. AFLP primarily distinguished *Y. enterocolitica* strains according to their biotype, with strains dividing into two distinct clusters: cluster A, comprising largely the putatively pathogenic biotypes (BT2 to -4), and cluster B, comprising the putatively nonpathogenic biotype 1A strains and a single BT1B isolate. Within these two clusters, subclusters formed largely on the basis of serotype. However, AFLP profiles also allowed differentiation of strains within these serotype-related subclusters, indicating the high discriminatory power of the technique for *Y. enterocolitica*. Investigation of the relationship between strain AFLP profile and host confirmed that pigs are, and provides further proof that sheep may be, potential sources of human infection with putatively pathogenic strains. However, the results suggest that some strains causing human disease do not come from veterinary sources identifiable at this time. The distribution of some BT1A isolates within cluster A raises questions about the relationship between virulence potential and biotype.**

*Yersinia enterocolitica* is a gram-negative enteric human pathogen that can cause a range of human diseases from mild diarrhea to mesenteric lymphadenitis (5). Disease is generally self-limiting, but severe disease can occur, especially in immunocompromised patients. In some groups of patients, postinfectious sequelae, such as arthropathies, are not uncommon. Experimental models suggest that infection involves invasion of the gastrointestinal epithelium. Invading organisms then escape from the epithelial cell via the basolateral membrane. The bacteria then propagate in the lamina propria, where the production of a variety of virulence factors aids subversion of the host immune response.

Approximately 300 cases of yersiniosis per annum in England and Wales are reported to the Communicable Disease Surveillance Centre. This incidence is likely to be an underestimate given that diarrheic stool samples are infrequently cultured and *Y. enterocolitica* is difficult to isolate from such specimens. In North America and some regions of Europe, the incidence and relevant importance of yersiniosis, as a cause of enteric disease, are considerably higher. Infections with *Y. enterocolitica* are apparently increasing worldwide (4). In some countries, such as Belgium, Holland, Canada, and parts of Germany, enteropathogenic *Yersinia* even rivals *Salmonella* as a cause of acute gastroenteritis (4, 5, 9).

There is considerable phenotypic diversity within *Y. enterocolitica*. Strains are generally grouped according to biotype (BT) and serotype. These groupings correlate with putative human pathogenicity and ecological and geographical distribution. Isolates belonging to biotype 1A (BT1A) are regarded as avirulent due to the absence of clinical effects in a mouse model and the lack of the pYV plasmid (4). Strains of biotypes 1B, 2, 3, 4, and 5 possess the pYV plasmid. BT1B isolates are considered highly pathogenic due to their lethality in the mouse model and are frequently associated with severe, potentially fatal diseases in humans (5). In contrast, strains of BT2 to -5 have relatively low pathogenicity in the mouse model. Most of the United Kingdom isolates belong to serotype O:9 (BT3), serotype O:5,27 (BT3), or serotype O:3 (BT4). In other geographical regions, different serotypes may predominate; for example, in the United States, serotype O:8 (BT1B) was the most predominant isolate, though it is now being superseded by serotype O:3 (BT4) (5).

The major routes of human infection are presumed to be food borne (4, 11, 12, 14). Because *Y. enterocolitica* has the ability to multiply at temperatures approaching 0°C (5), this pathogen can cause specific problems in the refrigerated food chain. The major food-borne sources of human infections remain unclear, although consumption of contaminated porcine products has been linked with disease (12). Most livestock species may be colonized asymptomatically. A recent national survey of livestock at the time of slaughter in Great Britain indicated that 6% of cattle, 13% of sheep, and 26% of pigs are colonized with this organism. In an attempt to determine the relative risks to human health from such sources, a phenotypic comparison of strains collected over the same time period from livestock and humans with disease was recently undertaken (23). This study concluded that biotyping and serotyping were

* Corresponding author. Mailing address: Department of Food and Environmental Safety, Veterinary Laboratories Agency, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom. Phone: 44 1932 357547. Fax: 44 1932 357595. E-mail: d.newell@vla.defra.gsi.gov.uk.

TABLE 1. Breakdown of the sources, biotypes, and serotypes of strains analyzed by AFLP

| Source | Biotype | Serotype | No. of isolates |
|---|---|---|---|
| Pig | BT4 | O:3 | 9 |
| Human | BT4 | O:3 | 7 |
| Human | BT3 | O:9 | 6 |
| Pig | BT3 | O:9 | 6 |
| Pig | BT3 | O:? | 4 |
| Pig | BT3 | O:5,27 | 4 |
| Sheep | BT3 | O:5,27 | 3 |
| Pig | BT1A | O:5 | 3 |
| Cattle | BT1A | O:19,8 | 3 |
| Human | BT1A | O:6,30 | 3 |
| Human | BT1A | O:5 | 3 |
| Cattle | BT3 | O:5,27 | 2 |
| Pig | BT4 | O:5,27 | 2 |
| Sheep | BT1A | O:6,30 | 2 |
| Sheep | BT1A | O:5 | 2 |
| Sheep | BT1A | O:19,8 | 2 |
| Pig | BT1A | O:6,30 | 2 |
| Pig | BT2 | O:9 | 1 |
| Cattle | BT1A | O:6,30 | 1 |
| Sheep | BT2 | O:9 | 1 |
| Sheep | BT4 | O:3 | 1 |
| Sheep | BT1A | O:4,32 | 1 |
| Human | BT1A | O:? | 1 |
| Human | BT1B | O:19 | 1 |

insufficiently discriminatory for such epidemiological investigations.

Recently the value of molecularly based typing methods has been clearly demonstrated for a number of bacterial pathogens. Some of these techniques, such as pulsed-field gel electrophoresis (PFGE), have been applied to the typing of *Yersinia enterocolitica* (10, 13, 22) with varying degrees of success. Recently the technique of amplified fragment length polymorphism (AFLP) has been developed for typing bacteria and shown to be potentially more discriminatory than existing methods (17, 21, 24). In this study, the AFLP technique has been modified for *Yersinia enterocolitica* and used to type selected cattle, sheep, and pig strains recovered during the national abattoir survey and human disease isolates collected over the same time period.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Human *Y. enterocolitica* strains from England and Wales were collected by the Health Protection Agency during 1999 to 2000. Additional human strains from an Intestinal Infectious Disease study, carried out by the Food Standards Agency in England and Wales during 1996 (30), were also investigated. These strains were kindly supplied by Mike Hudson (Health Protection Agency, Porton Down, United Kingdom). Strains from cattle, sheep, and pigs were isolated, as previously described (23), during a national survey of enteric pathogens present in livestock at slaughter, carried out by the Department for Food, Environment, and Rural Affairs in Great Britain during 1999 to 2000. Seventy human and veterinary strains (Table 1) were selected to represent a cross section of the serotypes and biotypes of all *Y. enterocolitica* strains detected in the previous phenotyping study (23). Five *Yersinia rohdei* and two *Yersinia frederiksenii* isolates recovered during the study were used as controls. All strains were stored at −80°C after isolation and were cultured on 10% sheep blood agar plates at 28°C for 18 h under aerobic conditions prior to investigation.

**Biotyping/serotyping of isolates.** Isolates were biotyped and serotyped by the Laboratory of Enteric Pathogens, Health Protection Agency, Colindale, London, United Kingdom, using the modified scheme of Wauters (5). Isolates possessing a nontypeable serotype were termed O:?.

**Isolation of chromosomal DNA.** *Yersinia* cultures were grown overnight on 10% blood agar plates at 28°C. Bacterial cells were removed from the plates using a sterile cotton swab and washed with 1 ml sterile phosphate-buffered saline. Chromosomal DNA was extracted by using the Gentra Puregene DNA purification kit (Flowgen) and following the manufacturer's protocol for extraction of DNA from gram-negative bacteria. The concentration of DNA was determined spectrophotometrically at $A_{260}$ as described previously (27), and the sample was diluted in sterile distilled $H_2O$ to give a final concentration of 50 μg $ml^{-1}$.

**AFLP analysis.** AFLP analysis was performed using the restriction enzymes BamHI and BspDI (New England Biolabs, Herts, United Kingdom). Briefly, 3 μl of chromosomal DNA was digested in a 20-μl reaction mixture at 37°C for 2 h and then ligated using T4 DNA ligase (New England Biolabs) in the presence of restriction half-site specific adaptors (Bam [5′-GATCGACAGTGTACTCTAG TC-3′] and Bsp [5′-CGGACTAGAGTACACTGTC-3′]). An aliquot of ligated products was then subjected to selective PCR using a 5′ fluorescently labeled BamHI primer containing an additional cytosine at the 3′ end (5′-GAGTACA CTGTCGATCC-3′) and a BspDI primer containing an additional thymidine at the 3′ end (5′-GTGTACTCTAGTCCGAT-3′). Litigation reaction products were diluted 1:10, and 2 μl was used as a template in the PCR. PCRs were performed in 50-μl reaction mixtures containing 5 μl 10× PCR buffer, 2.5 mM $MgCl_2$, 13 ng of each primer, 100 μmol deoxynucleoside triphosphates, and 5 U *Taq* polymerase. PCR conditions were as follows: initial denaturation at 94°C for 4 min; 25 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 90 s; and a final extension at 72°C for 10 min. PCR products were analyzed via electrophoresis on an 8% denaturing polyacrylamide sequencing gel using an ABI 373A automated DNA sequencer. Bionumerics software (Applied Mathematics, Kortrijk, Belgium) was used to analyze the data, and patterns were clustered using the Pearson correlation method with a 1% tolerance window. Results are presented as a dendrogram reflecting the genetic homology between isolates (expressed as a percentage).

## RESULTS

**Development and validation of the AFLP method.** The combination of restriction enzymes BamHI and BspDI resulted in bands ranging from 35 bp to 500 bp. The reproducibility of the method was assessed using individual preparations of a single isolate on multiple gels ($n = 3$). The banding pattern was highly reproducible, with a similarity of at least 95% within each gel and 90% between gels.

Different *Yersinia* species demonstrated substantial differences in AFLP patterns (Fig. 1). For example, using this method, the similarity of *Y. rohdei* to *Y. enterocolitica* was only 15%, and *Y. frederiksenii* was only 10% similar to either *Y. rohdei* or *Y. enterocolitica*. Interestingly, bands which were common to all strains of *Y. enterocolitica* were absent in *Y. rohdei* and *Y. frederiksenii*. Similarly, bands common to *Y. frederiksenii* strains were absent in *Y. rohdei*.

**AFLP typing of *Y. enterocolitica* strains.** Seventy human and veterinary isolates, selected on the basis of previously identified (23) phenotypic characteristics, were typed by the AFLP method developed. These strains exhibited a wide diversity in AFLP patterns (Fig. 1) but clustered into two broad groups, clusters A and B. Cluster A primarily comprised strains of biotypes 2, 3, and 4, while cluster B contained only biotype 1 strains. The percentage of similarity between these two clusters was only 41.15%; however, isolates in subclusters within cluster A ranged from 66.8% to 95.2% similarity. Likewise, isolates in subclusters within cluster B ranged from 68.6% to 83.4% similarity. Closer examination of the AFLP patterns suggested the presence of a small number of bands consistently associated with the differentiation of cluster-A and cluster-B isolates.

Within clusters A and B, subclusters were also observed

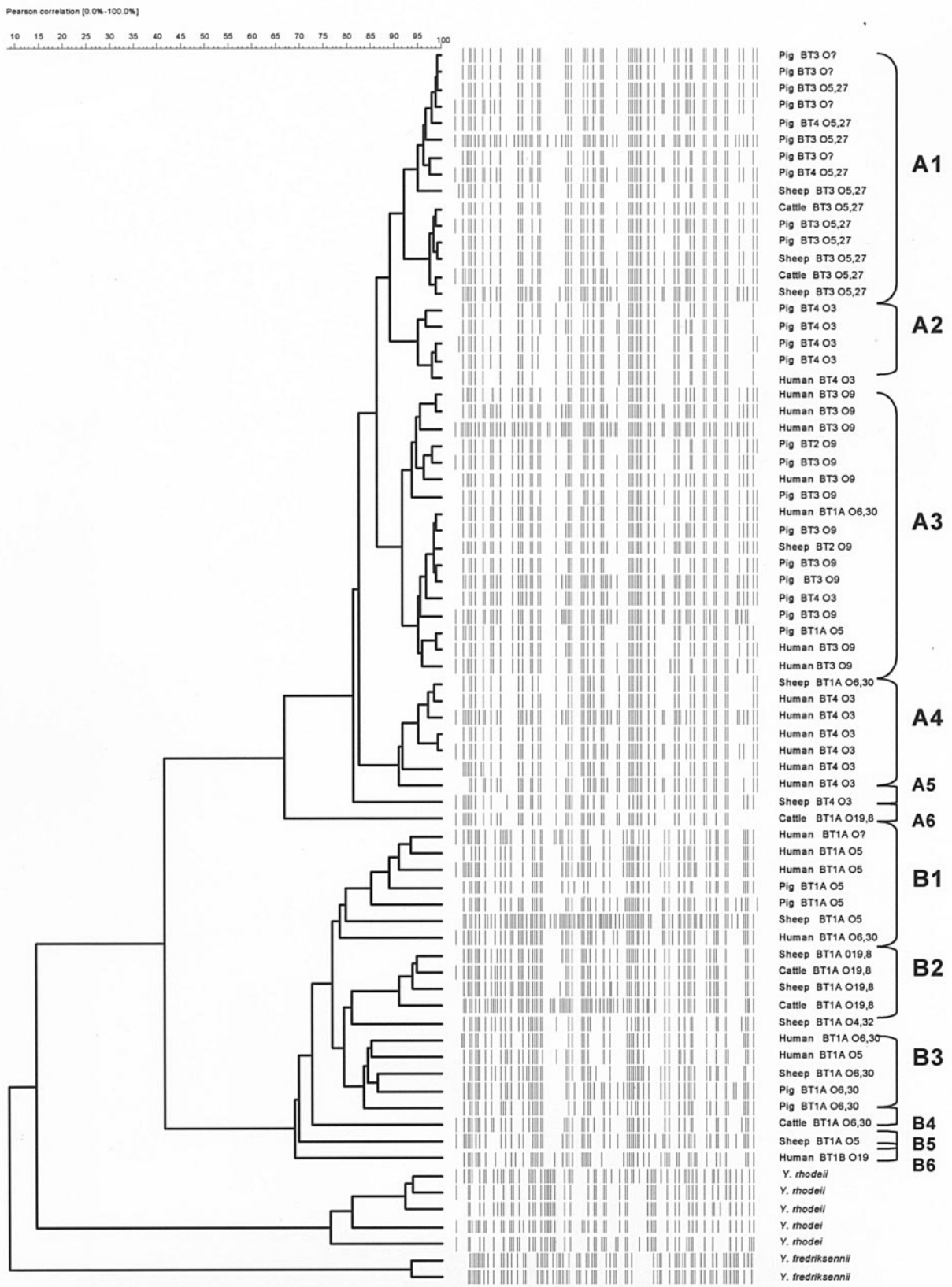


FIG. 1. Dendrogram of AFLP patterns of 70 *Y. enterocolitica* strains. Each cluster of isolates is labeled to the right of the dendrogram. Strains were isolated from sheep, cattle, pigs, or humans as indicated. The biotype (BT) and serotype of each strain are also given. Also included as controls are five *Y. rohdei* strains and two *Y. frederiksenii* strains. Cluster group numbers are given on the right.

TABLE 2. Summary of AFLP cluster types for *Y. enterocolitica* strains

| Cluster group | Source, biotype, and serotype | No. of isolates |
|---|---|---|
| A1 | Pig BT3 O:? | 4 |
| | Pig BT3 O:5,27 | 4 |
| | Pig BT4 O:3 | 4 |
| | Sheep BT3 O:5,27 | 3 |
| | Cattle BT3 O:5,27 | 2 |
| | Pig BT4 O:5,27 | 2 |
| A2 | Pig BT4 O:3 | 4 |
| | Human BT4 O:3 | 1 |
| A3 | Human BT3 O:9 | 6 |
| | Pig BT3 O:9 | 6 |
| | Pig BT2 O:9 | 1 |
| | Sheep BT2 O:9 | 1 |
| | Pig BT4 O:3 | 1 |
| | Human BT1A O:6,30 | 1 |
| | Pig BT1A O:5 | 1 |
| A4 | Human BT4 O:3 | 6 |
| | Sheep BT1A O:6,30 | 1 |
| A5 | Sheep BT4 O:3 | 1 |
| A6 | Cattle BT1A O:19,8 | 1 |
| B1 | Human BT1A O:5 | 2 |
| | Pig BT1A O:5 | 2 |
| | Sheep BT1A O:5 | 1 |
| | Human BT1A O? | 1 |
| | Human BT1A O:6,30 | 1 |
| B2 | Sheep BT1A O:19,8 | 2 |
| | Cattle BT1A O:19,8 | 2 |
| | Sheep BT1A O:4,32 | 1 |
| B3 | Pig BT1A O:6,30 | 2 |
| | Human BT1A O:6,30 | 1 |
| | Sheep BT1A O:6,30 | 1 |
| | Human BT1A O:5 | 1 |
| B4 | Cattle BT1A O:6,30 | 1 |
| B5 | Sheep BT1A O:5 | 1 |
| B6 | Human BT1B O:19 | 1 |

(Fig. 1; Table 2). These subclusters were associated with phenotypic properties. Cluster A divided into six subclusters (A1 to A6). Subcluster A1 ($n = 15$) comprised 11 O:5,27 strains (9 of which were BT3 and 2 of which were BT4) and 4 strains that were nonserotypeable but were all BT3. Isolates representing subcluster A2 ($n = 5$) were all serotype O:3 (BT4). Subcluster A3 ($n = 17$) was slightly more phenotypically heterogeneous, with 14 serotype O:9 isolates (12 of which were BT3 and 2 of which were BT2), 1 serotype O:3 (BT4) isolate, and 2 BT1A isolates of serotypes O:6,30 and O:5. Subcluster A4 ($n = 7$) comprised isolates of all the same phenotype (O:3 [BT4]), except for one that was serotype O:6,30 (BT1A). Subclusters A5 and A6 contained only one strain each of serotype O:3 (BT4) and serotype O:19,8 (BT1A), respectively.

Cluster B ($n = 20$) contained 19 BT1A strains of differing serotypes (Table 2). The remaining isolate was BT1B (serotype O:19). The degree of heterogeneity within the AFLP patterns in cluster B was greater than that observed for cluster A. Cluster B also subdivided into six subclusters (B1 to B6) (Fig. 1). Subcluster B1 ($n = 7$) contained five isolates of serotype O:5, one isolate of serotype O:6,30, and one nonserotypeable isolate. Subcluster B2 ($n = 5$) contained four strains of serotype O:19,8 and one of serotype O:4,32. All but one isolate of subcluster B3 ($n = 5$) belonged to serotype O:6,30. Subclusters B4 and B5 contained only one isolate each, of serotypes O:6,30 and O:5, respectively. Finally, subcluster B6 contained the only isolate of biotype 1B (O:19) in the collection. This BT1B strain was highly related to the BT1A strains and had less than 1% difference in relatedness to the subcluster B5 isolate.

There were no clear associations of host sources with AFLP patterns. Interestingly, six of the seven human BT4 O:3 strains were clustered together in subcluster A4 and had patterns distinctly different from those of the four pig BT4 O:3 strains that clustered together in subcluster A2. In contrast, the human BT3 O:9 strains in the collection clustered closely with the pig strains of the same phenotype.

## DISCUSSION

The importance of *Y. enterocolitica* strains in food-producing animals as a source of human disease remains debatable. The comparison of phenotypes has contributed significantly to our understanding of similar problems for other food-borne bacterial pathogens, such as salmonellae. However, previous comparison by routine phenotypic methods of *Y. enterocolitica* strains isolated over the same period in Great Britain, either from a survey of livestock at slaughter or from humans presenting with clinical symptoms, failed to identify any correlation between host and pathogenic potential (23). One possible explanation for this was the lack of discrimination provided by such phenotypic techniques. Recently genotypic approaches have been widely adopted to improve the discriminatory power of such comparisons. A variety of DNA-based typing methods have been developed previously for typing of *Y. enterocolitica*, including PFGE (2, 10, 22), ribotyping (20), and restriction enzyme analysis of virulence plasmid pYV (REAP) (16) or of chromosomal DNA (REAC) (18). Several of these methods have technical problems, which have generally precluded their adoption in reference laboratories. For example, REAC generates too many bands, while ribotyping produces too few bands, for appropriate levels of discrimination. In addition, typing systems based on plasmids are susceptible to the effects of environmentally mediated plasmid loss (19). Most recently, PFGE has been routinely employed to genotype *Y. enterocolitica*, and several of these studies have demonstrated an association between PFGE patterns and serotypes (6, 25, 26). However, the levels of discrimination of PFGE patterns within serotypes are limited, rendering additional differentiation of *Y. enterocolitica* impractical compared to that for other bacterial species such as *Escherichia coli* (1).

Most recently AFLP has been used for other enteric bacterial pathogens to compare strains from veterinary and human sources (17, 21, 24). In order to validate the use of AFLP as a typing technique for *Y. enterocolitica*, a selection of strains of biotypes 1A, 2, 3, and 4 were analyzed alongside isolates of *Y.*

*frederiksenii* and *Y. rohdei*. The resulting dendrogram (Fig. 1) revealed variable genetic distances among the different *Yersinia* species. This is consistent with other studies (7) and confirmed that AFLP could successfully differentiate between *Y. enterocolitica* and non-*Y. enterocolitica* members of the genus *Yersinia*.

AFLP analysis of the *Y. enterocolitica* isolates resulted in formation of a dendrogram composed of two distinct clusters (Fig. 1). The first cluster, cluster A, contained primarily isolates belonging to biotypes 2, 3, and 4. The second cluster, cluster B, contained isolates belonging to BT1A as well as the one BT1B strain tested. Because biotypes 2 to 5 are generally considered pathogenic while biotype 1A is nonpathogenic, these findings suggest that AFLP distinguishes primarily according to the pathogenicity of the biotype. Because AFLP is based on the analysis of chromosomal DNA, it is unlikely that the presence or absence of the pYV virulence plasmid of *Y. enterocolitica* could have been the distinguishing factor in this clustering effect. In addition to pYV, BT1A isolates also lack a number of chromosome-based virulence determinants, such as *ail*, *myfA*, and the enterotoxin genes *ystA* and *ystB* (15, 29). However, it is unlikely that such small genetic differences would result in such diversity (only 41.15% similarity) between clusters A and B and could not account for the association between AFLP pattern and serotype.

*Y. enterocolitica* is reported to be a highly homogeneous species (6, 16, 18), and AFLP analysis has confirmed a high degree of clonality, at least in relation to serotype. Isolates of serotype O:3 (subcluster A2) were the most clonal, with 95.23% identity found among the strains tested. This confirms previous observations using other approaches (3, 18). Similarly, isolates of serotype O:5,27 (subcluster A1) and serotype O:9 (subcluster A3) had 92.33% and 91.76% identity, respectively. Interestingly, the degree of clonality in cluster B was lower than that in cluster A, indicating that BT1A strains possessed greater heterogeneity. Najdenski et al. (25) also noted that when compared by PFGE, pathogenic serotypes were more clonal than nonpathogenic serotypes.

The AFLP technique developed has been used to investigate the relationship between genotype and host source. Interestingly, isolates from pigs, cattle, and sheep clustered with human isolates, suggesting that livestock species can be a reservoir for human yersiniosis. For example, six human and six pig isolates of serotype O:9 (BT3) clustered together (subcluster A3). Further evidence that pigs may be a source of *Y. enterocolitica* was shown in cluster A2, where one human isolate (O:3 [BT4]) clustered with four pig isolates of the same biotype/serotype. Epidemiological studies have previously indicated that the consumption of reticuloendothelial tissue from uncooked or undercooked pork constitutes a significant risk of disease (5, 28). The genotypic link between porcine and human isolates has been indicated previously using restriction fragment length polymorphism analysis (2).

The evidence provides further proof that sheep may also be a source of human infection. For example, in subcluster A4, 6 human O:3 (BT4) isolates clustered with one sheep isolate of the same biotype; however, the link between the two groups is tenuous and requires further investigation with a larger data set. Similarly, because of the lack of human isolates with a biotype matching that of the predominant cattle-pathogenic strains (BT3 [O:5,27]), no conclusions could be drawn about the relationship between human disease and yersinia carriage in bovines.

AFLP appears to contribute additional information to that obtained by serotyping and biotyping, presumably reflecting its higher discriminatory power. For example, two distinct populations of BT4 O:3 isolates were identified: one in subcluster A2, which contains five BT4 O:3 strains, four of which are porcine and one human; and a second group comprising six BT4 O:3 strains, all of human origin, in subcluster A4. Similar results have been reported previously using multilocus enzyme electrophoresis (8). These data suggest that at least some of the human BT4 O:3 strains are unrelated to the BT4 O:3 strains carried by pigs. Potential sources of these non-pig-related strains are as yet unidentified.

Interestingly, AFLP identified four BT1A isolates, which grouped in cluster A, the putatively pathogenic group. Clearly the characterization of such anomalous strains requires further investigation, as does the location of the single BT1B isolate in cluster B, comprising putatively nonpathogenic strains. The relationships between phenotype/genotype and virulence potential in such strains are currently under investigation using a variety of surrogate in vitro models.

In conclusion, the value of AFLP for distinguishing between strains of *Y. enterocolitica* has been demonstrated. This technique, when used to compare strains from humans, cattle, sheep, and pigs, has provided some novel evidence about the potential veterinary sources of human yersiniosis. Moreover, although the analysis of profiles clearly shows that AFLP genotype clusters predominantly reflect strain serotype and biotype characteristics, anomalous strains occur within these clusters, which raises questions about the pathogenic potential of such strains.

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