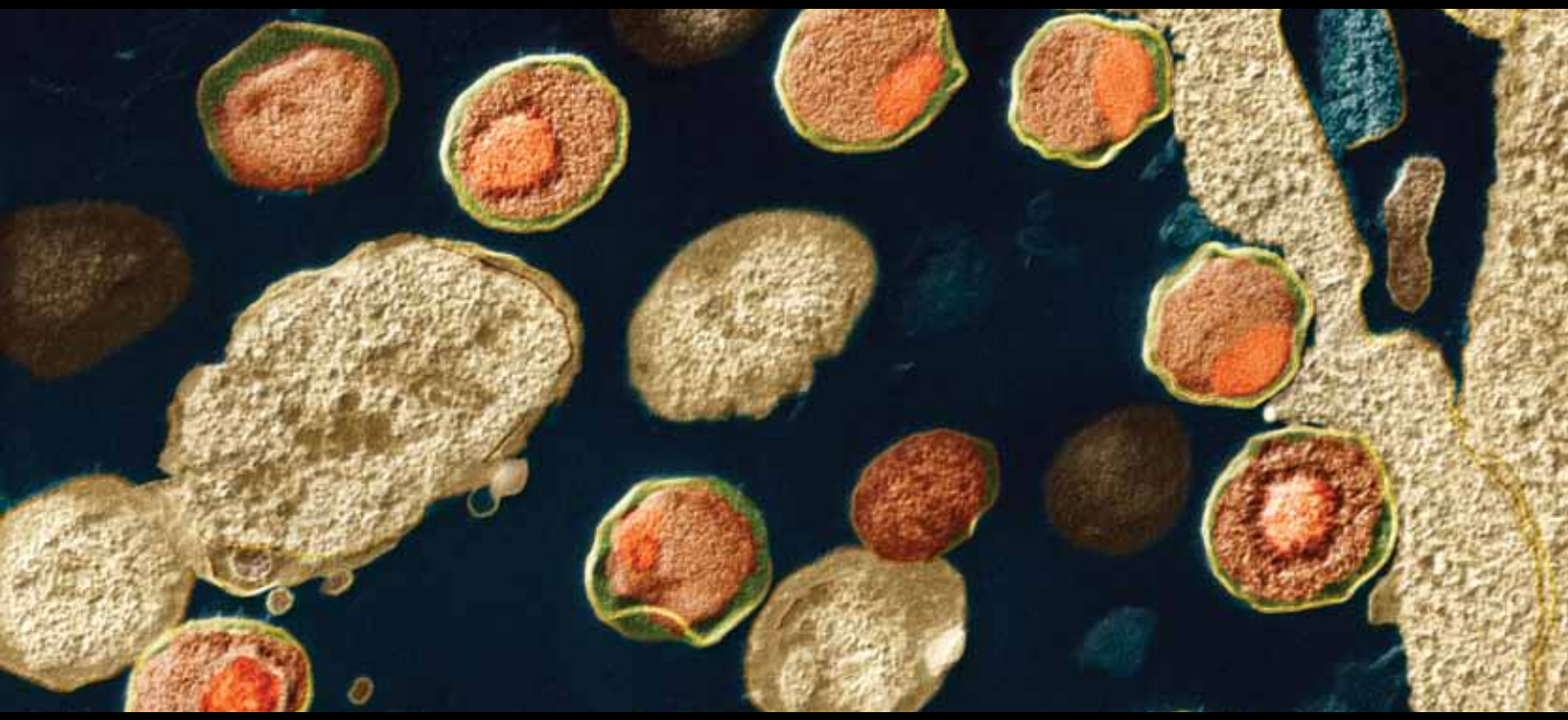
Journal of Pathogens

Yersiniosis and Food Safety

Guest Editors: Latiful Bari, Dike O. Ukuku, Kenji Isshiki, Ramesh C. Ray, and Didier Montet



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Editorial Board

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Editorial

Yersiniosis and Food Safety

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This special issue of Journal of Pathogens was designed to share some of the interested scientific studies published on yersiniosis, a foodborne outbreaks associated with consumption of food contaminated with *Yersinia*. In this issue, the focus was on yersiniosis-related foodborne illnesses, behavior of *Yersinia* in foods, incidence, persistence, survival, or growth, outbreaks and surveillance, zoonosis virulence and pathogenesis, detection/identification, mechanisms to grow in foods, and public health. *Yersinia* belongs to the *Enterobacteriaceae* family and is often isolated from clinical specimens. Three *Yersinia* strains, namely, *Y. enterocolitica, Y. pseudotuberculosis,* and *Y. pestis,* are pathogenic to humans and are widespread among various animal species and in the environment. They are transmitted to humans by the oral route and cause intestinal symptoms such as abdominal pain, diarrhea, and fever. These species are found all over the world, with a higher incidence in temperate and cold environments.

In this special issue, behavior of *Yersinia enterocolitica* in foods, their incidences, possible route of contamination, persistence, factors that influence the survival, or growth in food, soil, and water are reviewed by Bari et al.

The epidemiology, outbreaks and surveillance, and zoonosis of *Yersinia* spp. and their current status in different foods and environments are discussed by A. Rahman et al.

The molecular insight of virulence of *Yersinia enterocolitica*, mode of transmission of virulence, and their factors are covered by Y. Sabina et al.

The pathogenesis of *Yersinia enterocolitica* and *Y. pseudotuberculosis* in human yersiniosis, their genomics, mechanisms of infection, and host responses including the current state of surveillance, detection, and prevention of yersiniosis, are presented by C. L. Galindo et al.

The virulence plasmid (pYV) associated with the expression of phenotypic virulent in pathogenic *Yersinia* species and procedure to monitor the presence of virulence plasmid in *Y. Pestis* during storage and a convenient culture method for monitoring the presence of virulent plasmid in food are discussed by S. Bhaduri and J. L. Smith.

A highly sensitive, specific, and accurate selective chromogenic culture plate method that has been developed for detecting pathogenic *Y. enterocolitica* from pig tonsils was discussed by M. Denis et al.

H. Fukushima et al. reviewed and discussed the commercially available conventional and PCR-based procedures for specific detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in foods.

J. Gui and I. R. Patel reviewed and discussed the recent advances in molecular technologies and their application in detecting pathogenic *Yersinia* in foods.

R. Das et al. reported in their research article the presence of a novel single-stranded DNA in *Yersinia frederiksenii* and their genomic analysis, and they found that enzyme might be responsible for the transposition of this novel retron element.

In the last reviewed article, S. N. Aziz and K. M. S. Aziz discussed the theoretical modeling to avoid exposure of *Yersinia enterocolitica* infections in foods.

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Research Article

Averting Behavior Framework for Perceived Risk of *Yersinia enterocolitica* Infections

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The focus of this research is to present a theoretical model of averting actions that households take to avoid exposure to *Yersinia enterocolitica* in contaminated food. The cost of illness approach only takes into account the value of a cure, while the averting behavior approach can estimate the value of preventing the illness. The household, rather than the individual, is the unit of analysis in this model, where one household member is primarily responsible for procuring uncontaminated food for their family. Since children are particularly susceptible and live with parents who are primary decision makers for sustenance, the designated household head makes the choices that are investigated in this paper. This model uses constrained optimization to characterize activities that may offer protection from exposure to *Yersinia enterocolitica* contaminated food. A representative household decision maker is assumed to allocate family resources to maximize utility of an altruistic parent, an assumption used in most research involving economics of the family.

Yersiniosis remains a public health hazard due to exposure to contaminated food and human to human or zoonotic infections. *Yersinia enterocolitica* is an important cause of yersiniosis in humans and animals; its epidemiology remains yet to be fully understood and exposure to it is a growing food safety concern [1–5]. There are a number of recent reviews published on specific aspects of *Y. enterocolitica*, and while some of these studies investigate incidence rates, true incidence in developed and developing countries remain unknown [1, 6–10]. One of the most frequent outcomes of *Y. enterocolitica* is possibly diarrhea as exemplified by a study in Poland [11]. A study on methods of monitoring trends in incidence of foodborne diseases in the United States is a welcome instrument in the estimation of incidence of *Y. enterocolitica* and other pathogens [12]. Studies of incidence, combined with studies investigating behaviors of individuals responding to information of incidence and risk levels of *Y. enterocolitica* can be useful for public health mitigation policies. In this paper we discuss a behavioral model with a focus on avoiding health hazards associated with exposure to *Y. enterocolitica*. The paper is theoretical and the conceptual model presented here is not showcased with data. The theoretical framework easily lends itself to application subject to availability of secondary data. One major thrust of the theoretical discussion revolves around the heuristic notion "an ounce of prevention is worth a pound of cure."

As infection from *Y. enterocolitica* is typically contracted through eating contaminated food, an examination of averting behavior may help identify the burden of disease better than the cost of illness approach. Any action taken by an individual to avoid an illness is considered averting behavior. Cost of illness is typically and widely used in public health policy analysis and includes both direct and indirect costs. Whether analyses incorporate direct or indirect costs, cost of illness studies focus on estimating the costs of cure. Direct costs take into account the cost of resources used to treat illness while indirect cost measures the value of resources foregone due to the illness. Cost of illness studies are generally considered underestimates as they do not take into account two things. Cost of illness studies do not take into account pain and suffering as one endures an illness.

Also, the notion that cost of illness studies provide a lower bound for the value of avoiding the illness is supported by the heuristic notion that a person is willing to pay significantly more to avoid an illness (values toward prevention) than to become ill in the first place (values toward cure).

Averting behavior models take into account any action or expenditures that individuals undertake to avoid an illness. Averting behavior models are protective expenditures or actions that individuals undertake to avoid exposure to any undesirable outcome (e.g., pollution, illness, death). This is an approach that says that the value of a small reduction in health state can in principle be measured by the amount an individual is willing to spend on some defensive or averting action to prevent it. The assumption in the averting behavior approach in this study is that people make choices in order to maximize their level of well-being when faced with increased health risks associated with exposure to contaminated food [13]. The notion here is that individuals present subjective individual preferences toward avoiding the illness. Their subjective individual valuations are measured and taken as given. The main hypothesis here is that individuals value their health and make optimizing choices to maximize their well-being subject to certain constraints. Assuming only monetary choices (averting behavior studies do not preclude individual actions, but in this note we are assuming expenditures only), individuals make these choices subject to their budget constraints. Also, individuals make these choices about risk mitigation without knowing whether they or a member of their household will be ill: choices are predicated on perceived risk of *Y. enterocolitica*.

Along these lines, we present a simple model of choice under uncertainty. Most behavioral economics use the notion of utility, where utility represents an individuals' level of well-being. In economic theory, individuals are assumed to take actions in order to maximize their level of well-being, and these actions are limited by their resource constraints. Here, we define Utility (U_i) to represent individual well-being. Utility (U_i) is assumed to be a function of wealth (W), health (H), perceived risk levels (r), and averting activities ($\propto$). The resource constraint that the individual faces is represented by W, which the reader can intuitively take to mean real wealth. W is what remains of individual wealth after costs of actions undertaken to avoid the risk are taken into consideration. If the level of action is represented by $\propto$, and if it costs the individual a price p per unit of the averting activity, we can represent the remaining amount of real wealth by removing the total cost of the averting activity $p \propto$ from initial wealth levels (W_0). Since individuals do not know whether they will be ill from contaminated food, their utility (of maintaining health and well-being) is not known with certainty. In order to deal with this, utility must be cast in a framework consistent with the probability of becoming ill [14]. Therefore, utility is weighted by probability of health state where the simplest possible health state is examined. Health states are indexed by *i,* where i goes from 1 to 2. Health states can theoretically be indexed by i going from 1 through n possible future states of health. For ease of exposition, i is suppressed to only two states indicating the probability that one can either be healthy ($i = 1$) or ill ($i = 2$) from *Y. enterocolitica* contaminated food. Also please note that wealth, health, and risk levels are themselves functions of the level of averting activity. The arguments for these variables are suppressed in the objective function for notational simplicity. Maximizing utility subject to the budget constraint is then expressed as follows:

$$\begin{aligned} \max_{\propto} \quad & \sum_{i=1}^{2} \pi_i U_i(W, H, r, \propto), \\ \text{Subject to} \quad & W = W_0 - p \propto, \end{aligned} \tag{1}$$

where π_i is probability of being in health state i, U_i is utility (well-being) in health state i, W is wealth, H is individual health, r is perceived ambient risk from *Y. enterocolitica,* $\propto$ is averting activity, W_0 is initial wealth level, and p is price of averting activity.

Assuming the simplest possible form of utility function (one in which additional utility from health, wealth, and from reducing risk are additive), we can take first-order conditions. Translated in discrete terms, we are finding the point at which an individual can choose the maximum amount of utility allowed by their budget constraint. In continuous terms, when we take the first-order conditions with respect to the averting activity $\propto$ and perceived risk reductions r, we find a simple efficiency condition:

$$\sum_{i=1}^{2} \pi \left(\frac{\partial U_i}{\partial \alpha} + \frac{\partial W}{\partial \alpha} + \frac{\partial r}{\partial \alpha} \right) = p. \tag{2}$$

The condition above simply states that averting activity will continue until the incremental benefits perceived from averting (left hand side) equal the incremental cost of averting (right hand side). This condition along with an individual's action to reduce risk levels (represented mathematically by first order conditions optimizing over reducing risk levels r) form the estimated value for averting *Y. enterocolitica.* The first order conditions over reducing risk level r are of the form:

$$\sum_{i=1}^{2} \pi \left(\frac{\partial U_i}{\partial r} + \frac{\partial W}{\partial r} + \frac{\partial r}{\partial r} \right) = 0. \tag{3}$$

Please see the appendix for derivations of the above equations. This first-order condition represents the individuals choice to reduce risk from contracting infection from *Y. enterocolitica.* Both conditions above intuitively represent individuals choice, and most importantly, value from averting infection from *Y. enterocolitica*. This paper does not apply the theoretical model above to data, primarily because no secondary data is available, and therefore we cannot comment on the value of using averting behavior versus cost of illness for *Y. enterocolitica.* While the empirical studies comparing WTP (willingness to pay) estimates with cost of illness (COI) estimates are few and far between, the comparisons that have been done show that WTP is at least 1.6 to 8.0 times larger than COI., [15–19]. One recent study [20] computed and compared willingness to pay for avoiding *shigellosis* to the cost of illness of *shigellosis.* The evidence on

whether WTP estimates are higher than COI estimates were mixed in the paper. Key messages from the study include the following:

> *"For evaluating the benefits of public programmes to control shigellosis, the use of the conventional and convenient ex post COI figures for adults instead of ex post WTP measures may yield acceptable measures of the welfare impacts of reducing disease risk.*
> *However, the use of ex post COI as the estimate of the welfare impact of risk-reducing policies is likely to underestimate the benefits of programmes to prevent shigellosis in children."*

While cost of illness approaches to prevent *Y. enterocolitica* in adults may suffice, public health mitigation policy makers may wish to focus on using methodologies such as averting behavior to estimate values for avoided illnesses in children. A direction for future research using the averting behavior model developed in this paper involves collecting primary data in order to test the theoretical model. A swift review of the studies cited, and future work with an applied averting behavior model may support the following notion "an ounce of prevention is worth a pound of cure, in the case of children." Perhaps this reflects the notion that in the case of adults, taking a calculated risk may be more palatable than in the case of children.

Appendix

Considering (1) we have the following.

Assuming an additively separable utility function the following first-order conditions with respect to the averting activity $\propto$ follow.

Let

$$EU = \sum_{i=1}^{2} \pi \left(\frac{\partial U_i}{\partial \alpha} + \frac{\partial W}{\partial \alpha} + \frac{\partial r}{\partial \alpha} \right). \tag{A.1}$$

Taking first-order conditions from the utility function with respect to $\propto$ yeilds

$$\frac{\partial EU}{\partial \propto} = \sum_{i=1}^{2} \pi \left(\frac{\partial U_i}{\partial \alpha} + \frac{\partial W}{\partial \alpha} + \frac{\partial r}{\partial \alpha} \right). \tag{A.2}$$

Taking first-order conditions from the budget constraint with respect to $\propto$ yeilds

$$\frac{\partial W}{\partial \propto} = p. \tag{A.3}$$

Equations (A.2) and (A.3) taken together represent the point of utility maximization where the slope of the budget constraint $\partial W/\partial \propto$ must be equal to the tangent to the utility function $\partial EU/\partial \propto$.

Therefore,

$$\sum_{i=1}^{2} \pi \left(\frac{\partial U_i}{\partial \alpha} + \frac{\partial W}{\partial \alpha} + \frac{\partial r}{\partial \alpha} \right) = p. \tag{A.4}$$

Equation (A.4) simply states that averting activity will continue until the marginal benefits perceived from averting (left-hand side) equal the marginal cost of averting (right-hand side). This condition along with first-order conditions optimizing over reducing risk levels r form the estimated value for averting *Y. enterocolitica*.

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Research Article

A Novel msDNA (Multicopy Single-Stranded DNA) Strain Present in *Yersinia frederiksenii* ATCC 33641 Contig01029 Enteropathogenic Bacteria with the Genomic Analysis of It's Retron

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Retron is a retroelement that encodes msDNA (multicopy single-stranded DNA) which was significantly found mainly in Gram-negative pathogenic bacteria. We screened *Yersinia frederiksenii* ATCC 33641 contig01029 for the presence of retroelement by using bioinformatics tools and characterized a novel retron-Yf79 on the chromosome that encodes msDNA-Yf79. In this study, we perceived that, the codon usage of retron-Yf79 were noteworthy different from those of the *Y. frederiksenii* genome. It demonstrates that, the retron-Yf79 was a foreign DNA element and integrated into this organism genome during their evolution. In addition to this, we have observed a transposase gene which is located just downstream of retron-Yf79. So, the enzyme might be responsible for the transposition of this novel retron element.

1. Introduction

For the past 21 years, it has been shown that some pathogenic Gram-negative bacteria strains contain genetic elements called retrons. Retron is a retroelement consisting of *msr*, which encodes the RNA part of msDNA, *msd*, which encodes the DNA part of msDNA, and the *ret* gene for reverse transcriptase (RT) [1]. The reverse transcriptase (RT) was originally discovered in virus [2] as an essential enzyme for the replication of retroviruses. Since the discovery of RT in myxobacteria [3] and *Escherichia coli* [4] an intriguing question have been raised concerning its origin and function in the prokaryotes [5].

The msDNA (multicopy single-stranded DNA) is composed of a small, single-stranded DNA, linked to a small, single-stranded RNA molecule. The 5′ end of the DNA molecule is joined to an internal guanine base (G) residue of the RNA molecule by a unique 2′, 5′-phosphodiester bond [6]. Since msDNA was originally discovered in the Gram-negative soil bacterium, *Myxococcus xanthus* [7] it was also isolated from aggregative adherence *E. coli* (AAEC) [8], a classical enteropathogenic *E. coli* (EPEC) [9] and more recently from *Vibrio cholerae* [10], *Salmonella enterica* serovar Typhimurium [5], *V. parahaemolyticus* and *V. mimicus* (Shimamoto T, 2003, unpublished data). Hence, RT might have a role in diversification of pathogenic bacteria genomes.

Although msDNAs have been isolated over the pathogenic Gram-negative bacteria, in this study we characterized a novel retron region by screening the complete genome sequence of *Yersinia frederiksenii* [11] which encodes *msr*, *msd* with a *ret* gene by best hits RT sequence similarity along with *V. cholerae, V. parahaemolyticus and S.* Typhimurium. These provide insight into the important roles of this mysterious element in these bacteria species.

2. Materials and Methods

2.1. Genomic Analysis of Retron-Yf79.

2.1.1. Sequence Retrieval. To determine the particular place of retron-Yf79, the complete nucleotide genome sequence of *Yersinia frederiksenii* ATCC 33641 contig01029 was retrieved from the national center for biotechnology information (NCBI) resource at (http://www.ncbi.nlm.nih.gov/) with the following accession number (AALE02000035) [11]. To investigate an evolutionary relationship among amino acid sequence of reverse transcriptases from *Y. frederiksenii*, *V. cholerae, V. parahaemolyticus* and *S.* Typhimurium; were collected from ExPASy proteomics server at (http://www.expasy.org/). In addition, the 16S ribosomal RNA (16S rRNA) nucleotide sequences of *Y. frederiksenii*, *V. cholerae, V. parahaemolyticus* and *S.* Typhimurium were collected from the kyoto encyclopedia of genes and genomes (KEGG) organism database available at GenomeNet server, Japan (http://www.genome.jp/) to observe the possible evolutionary scenario among those species.

2.1.2. Sequence Alignment. The genomic organization of *msd-msr* region of retron-Yf79 was determined according to their nucleotide sequences analyzing, that is, the presence of conserved region nucleotides with other *msr-msd* coding regions which have been isolated from various pathogenic bacteria- (*V. cholerae, V. parahaemolyticus* and *S.* Typhimurium) by using (ClustalW) program available at (http://www.genome.jp/tools/clustalw/), Japan [12]. To evaluate the similarity of RT-Yf79 with others RT-Vc95 from *V. cholerae* [10], RT-Vp96 from *V. parahaemolyticus* (Shimamoto T, 2003, unpublished data) and RT-St85 from *S.* Typhimurium [5], the alignment program was utilized at the site (http://www.genome.jp/tools/clustalw/) [12], after determining the best hit of RTs sequence similarity search by the BLAST program at NCBI Blast homepage (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi).

2.1.3. Structure Prediction and Codon Bias Analysis. The DNA and RNA secondary structures of msDNA-Yf79 were predicted by using the database-(http://www.ncrna.org/centroidfold/) [13]. The promoter sequence of retron-Yf79 was predicted on the basis of the conserved promoter sequences [14]. To appraise whether the retron is a foreign DNA element, the codon bias was carried out. The codon bias of retron-Yf79 and the whole organism genome was resolute by using codon usage database-(http://www.kazusa.or.jp/codon) [15].

2.1.4. Phylogenetic Analysis. To evaluate the origin and similarity of RT-Yf79 from *Y. frederiksenii*, phylogenetic tree was constructed by using other RTs from (*V. cholerae, V. parahaemolyticus* and *S.* Typhimurium). These amino acid sequences were aligned along with each other by using (ClustalW) at (http://www.genome.jp/tools/clustalw/), Japan [12]. The sequence alignment was performed under default conditions and the phylogenetic tree was constructed by the neighbor-joining method. The phylogenetic tree of 16S ribosomal RNAs was also constructed based on their nucleotide sequences by using same database available at (http://www.genome.jp/tools/clustalw/), Japan [12].

3. Results

3.1. The Structure of msDNA-Yf79. Analysis of *msd* nucleotide sequence showed that the DNA part of msDNA found in *Y. frederiksenii* is predicted to consist of 79 bases of a single-stranded DNA, and hence it was named as msDNA-Yf79, and the RNA part of msDNA-Yf79 consists of 70 bases encoded by *msr* gene of retron-Yf79 (Figure 1(a)). Furthermore, the guanine base (G) residue at position 12 of the RNA molecule branched out by a unique 2′, 5′-phosphodiester link (Figure 1(a)). The msDNAs isolated from other bacteria contains at least one mismatched base pair in their DNA stems which could be mutagenic [16, 17]. However, in this study we observed that the DNA structure of msDNA-Yf79 contains no any mismatched base pair as like as most of msDNAs were isolated from other pathogenic bacteria (Figure 1(a)). Further, the msDNA-Yf79 shared a number of conserved nucleotide sequences with other msDNAs (msDNA-St85,-Vc95 and -Vp96) (Figure 1), except thymine (T) at position 67 in DNA part of msDNA-Yf79 (Figure 1(a)).

3.2. Genomic Organization of Retron-Yf79. The retron-Yf79 consists of nucleotide sequence of about 2.8 Kb, and the retron element is transcribed from the −35 and −10 conserved promoter sequence located 5 bp upstream to the *msr-msd* coding region (Figures 2(a) and 2(b)). In addition, two open reading frames (ORFs) were located just downstream of *msr* and *msd* coding sequence, one is RT-Yf79 encoded retron-type reverse transcriptase having 310 amino acids, and another one is ORF-541 which encoded a putative ATP binding protein containing 541 amino acids (Figure 2(a)). The upstream and downstream regions of retron element also contained yfred0001_42820 gene that encoded a hypothetical protein (356 AAs) and Yred0001_42860 gene that encoded a transposase (308 AAs), respectively (Figure 2(a)).

3.3. Codon Usage of Retron-Yf79. To identify the origin of RT-Yf79 and ORF-541 genes in *Y. frederiksenii* genome, the codon usages were carried out. It revealed that the RT-Yf79 and ORF-541 genes used AAA codon for lysine with a frequency of 55% and 74%, respectively, but the *Y. frederiksenii* genome only used AAA codon for lysine with a frequency of 20% of the time (data not shown). Present observation suggested that the retron-Yf79 is a foreign DNA element and probably acquired in this organism chromosome from other ancestral species during their evolution times.

3.4. Comparative Study of RT-Yf79 with Other ret Genes from Different Pathogenic Bacteria. The RT-Yf79 encoded by the retron-Yf79 consists of 310 AA residues. Surprisingly, all retron RTs in pathogenic bacteria were shown to have

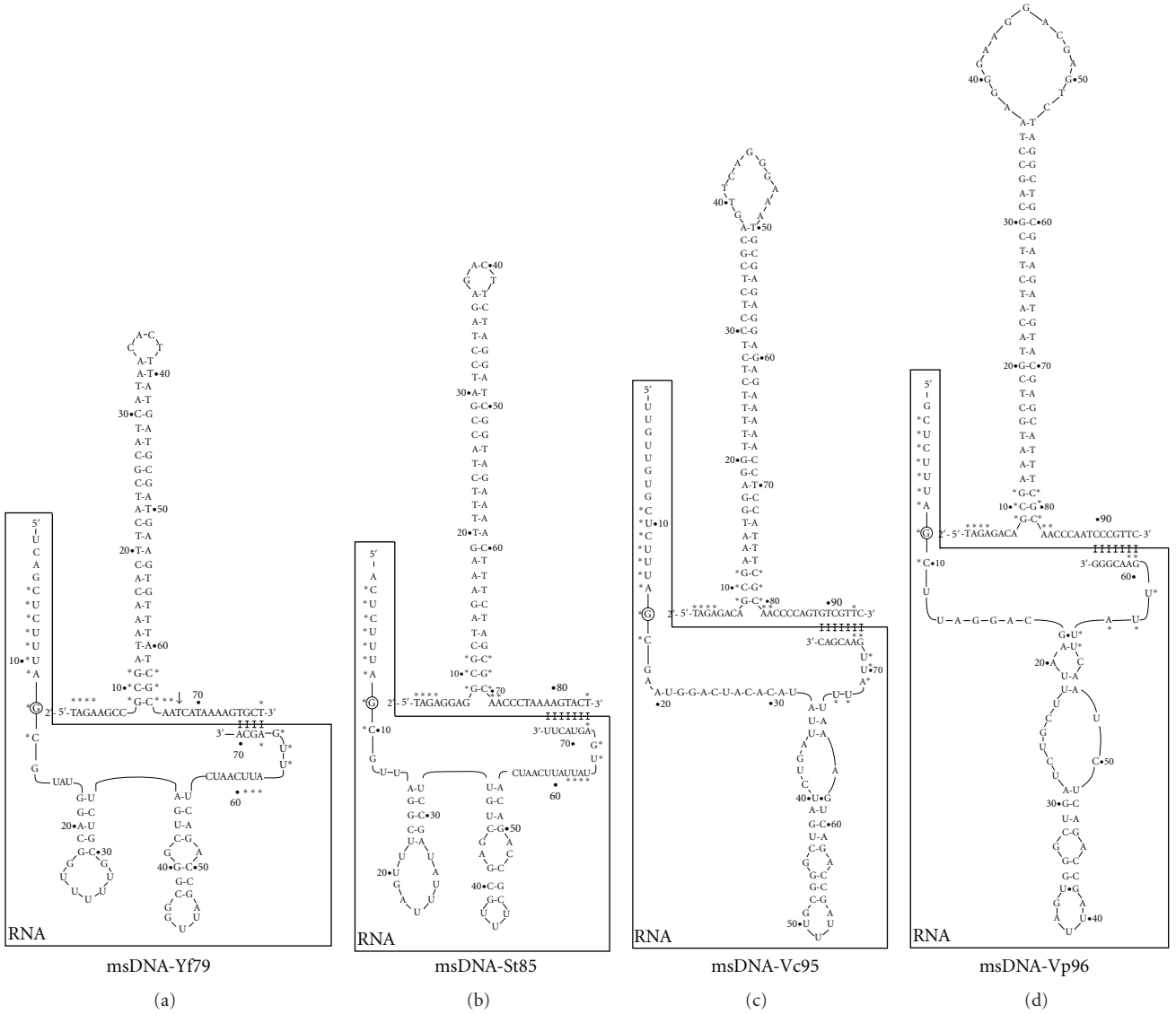

Figure 1: Possible secondary structures of multicopy single-stranded DNA (msDNAs) from pathogenic bacteria. (a) The branching guanine base (G) residue at position 12 in RNA portion of msDNA is circled and forming a 2′, 5′-phosphodiester bond (a). Both the DNA and RNA secondary stem loop structures were suggested on the basis of their sequences. The RNA portion was boxed and the numbers of RNA and DNA were begun from 5′ ends. The conserved nucleotides are indicated by stars in all msDNAs. (a) The msDNA-Yf79 is predicted from *Yersinia frederiksenii* [11], (b) msDNA-St85 is isolated from *S.* Typhimurium [5], (c) msDNA-Vc95 is from *V. cholerae* [10], and (d) msDNA-Vp96 is from *V. parahaemolyticus* (Shimamoto T, 2003, unpublished data). The arrow indicates thymine base (T) at position 67 in the DNA part of msDNA-Yf79 (a).

the highest identities to RT-Yf79: RT-Vc95 (from *V. cholerae,* 44% identity), RT-Vp96 (from *V. parahaemolyticus,* 45% identity), and RT-St85 (from *S.* Typhimurium, 43% identity) when these RTs were compared with each other by using multiple amino acids alignment (Figure 3). These four RTs shared approximately similar number of amino acids (Figure 3). In addition, they all shared a conserved domain along with each other (data not shown).

3.5. Phylogenetic Analysis of RTs and 16S Ribosomal RNA Gene Sequences. To observe the genomic diversity of *ret* genes and orthologous 16S ribosomal RNA genes (from *Y. frederiksenii*, *V. cholerae, V. parahaemolyticus* and *S.* Typhimurium) phylogenetic trees were constructed by using ClustalW at (http://www.genome.jp/tools/clustalw/), Japan [12] (Figure 4). The phylogenetic tree analysis showed a fundamental diversity among the *ret* genes in relation to the host bacteria (*Y. frederiksenii*) species as RT-Yf79 from *Y. frederiksenii* [11] was closely related to RT-Vp96 from *V. parahaemolyticus* (Shimamoto T, 2003, unpublished data) rather than to the RT-St85 from *S.* Typhimurium [5] and RT-Vc95 from *V. cholerae* [10] of pathogenic bacteria as RT-St85 was closely related to the RT-Vc95 (Figure 4(a)). Although both RT-Vc95 and RT-Vp96 were from *Vibrio* species, both were diverged from each other as they were closely related to RT-St85 and RT-Yf79, respectively (Figure 4(a)). The 16S

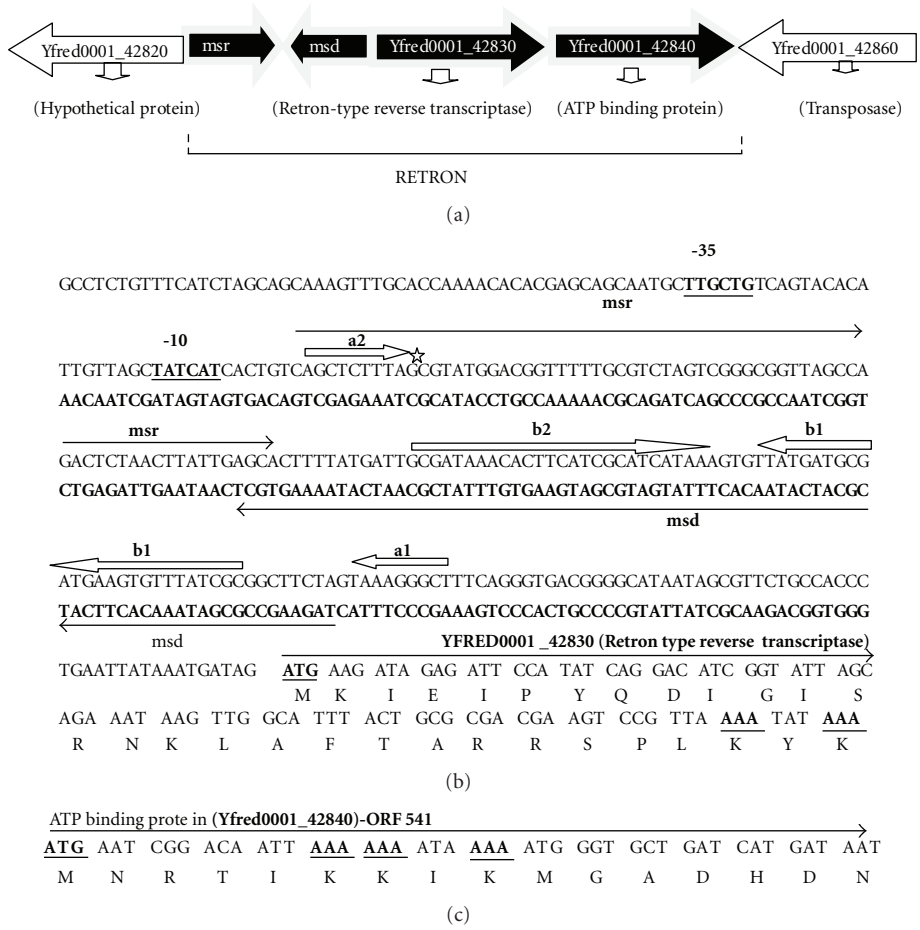

Figure 2: Genomic organization of retron-Yf79 in whole genome of *Yersinia frederiksenii* chromosome (a) and the *msr-msd* nucleotide sequence along with RT gene in (b): the −35 and −10 conserved promoter sequences are underlined and located at just upstream of *msr-msd* coding sequence. Inverted repeats, a1/a2 and b1/b2, are indicated by arrows, while the conserved guanine (the branching G) at the 12th position of the *msr* is shown by star on top of the G. The partial N-terminal amino acid sequences of both RT and ORF-541 are indicated, and the ATG (methionine) and AAA (lysine) are bold and underlined in (b and c).

ribosomal RNA phylogenetic analysis suggested that, these pathogenic bacteria genomes might acquire these retron elements during their evolution (Figure 4(b)).

4. Discussion

In this study, we demonstrated that a new msDNA-Yf79 exists in *Y. frederiksenii* ATCC 33641 contig01029 cell types and compared it's properties to that of St85 [5], Vc95 [10] and Vp96 (Shimamoto T, 2003, unpublished data). The retron-Yf79 was responsible for the production of msDNA-Yf79 in *Y. frederiksenii* Gram-negative pathogenic bacteria strain.

However, the gene organization of retron-Yf79 was similar to those found in *E. coli* (retron-Ec83 and -Ec78) [8, 9], that is, contained only two open reading frames (ORFs) in their retroelement. On the other hand, the gene organization of retron-Vc95 [10] and retron-Vp96 (Shimamoto T, 2003, unpublished data) were completely different as they contained a third ORFs. The msDNA-Yf79 has a sequence similarity to msDNA-St85, msDNA-Vc95 and msDNA-Vp96 as these msDNAs shared a number of highly conserved bases in their nucleotide sequences, indicating that they might be descended from a common origin (i.e., from a common ancestor). The presence of the conserved guanine base (G) at position 12 in RNA part of msDNA-Yf79 which involved in branch formation via a 2′, 5′-phosphodiater link in DNA-RNA complex (Figure 1(a)). Lima and Lim suggested that the fact that the mutation in guanine base (G) prevents msDNA synthesis and the primary product of reverse transcription may be a branched DNA-RNA compounds [9], which supports our observation.

Furthermore, it was quite interesting that stem structure of msDNA-Yf79 did not contained any mismatched base pair like most of the msDNA isolated from other pathogenic bacteria. Moreover, the codon usage of this retron element and also the phylogenetic analysis of RTs and 16S rRNA from pathogenic bacteria revealed that this retron was a foreign DNA element. The downstream of retron element-Yf79 contained a transposase gene indicating that this enzyme might be participated in transposition of this novel retron element in the genome.

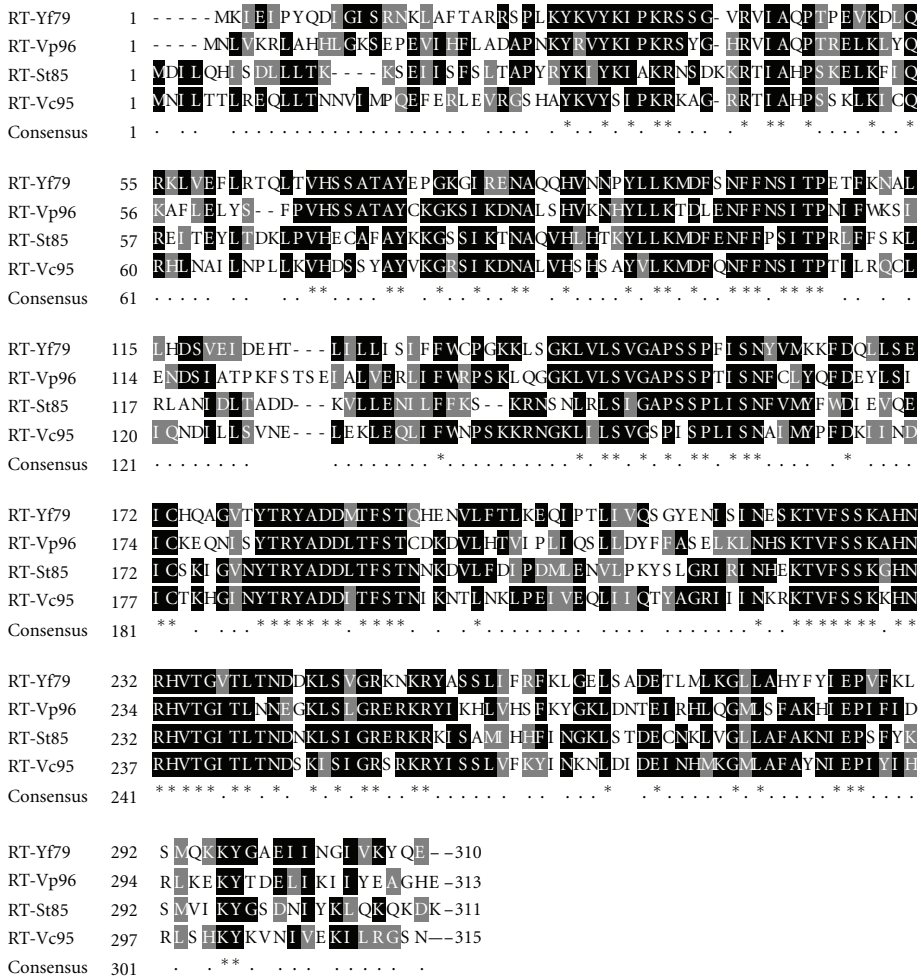

Figure 3: Comparison of the amino acids sequence alignment of the RT-Yf79 with the three highest identity RT sequences: RT-Vc95 (44% identity), RT-Vp96 (45% identity), and RT-St85 (43% identity). Amino acids conserved in all four RTs are marked with asterisks and black colors. Conserved and well-conserved amino acids residues are marked with dots and the number of amino acids of each RT was written at the end of the alignment.

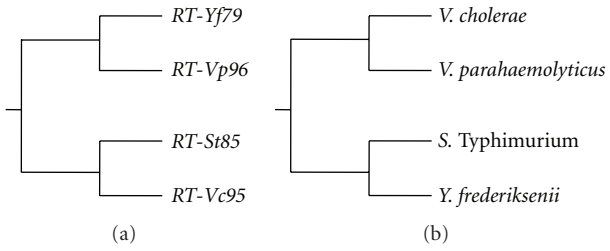

Figure 4: Phylogenetic trees among *Y. frederiksenii, V. cholerae, V. parahaemolyticus, and S.* Typhimurium based on the RT (a) and the 16S ribosomal RNA genes (b). The trees were constructed by using the neighbor-joining (NJ) method in the CluslalW program. The following ExPASy accession numbers for the RT sequences were used in the phylogenetic construction: *Y. frederiksenii* RT-Yf79-C4SUU2, *V. cholerae* RT-Vc95-Q9S1F2, *V. parahaemolyticus*-Q8L0W6, and *S.* Typhimurium- E7UVY4. The following GenomeNet accession numbers for the 16S rRNA sequences were used in the phylogenetic construction: *Y. frederiksenii*-NR_027544.1, *V. cholerae*-2614447, *V. parahaemolyticus*-1187490 and *S.* Typhimurium-1251767.

We resolved after consideration to look closely the nucleotide sequence of this retron-Yf79 in *Y. frederiksenii* because this organism has generated significant value in the role of pathogenicity. Functions of msDNA are still not clear. However, this DNA-RNA complex which was identified in Gram-negative pathogenic bacteria may support its role in the process of pathogenicity. In addition, retron element may play an essential role for adaptation of such bacteria in different stressful conditions by changing the expression of their regulatory social behavior under which conditions that expression is densely populated. Further experiment will be required for demonstrating the functions of msDNA, which may be opened a new arena in the process of pathogenicity or adaptation in stressful conditions.

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Review Article

Recent Advances in Molecular Technologies and Their Application in Pathogen Detection in Foods with Particular Reference to *Yersinia*

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Yersinia enterocolitica is an important zoonotic pathogen that can cause yersiniosis in humans and animals. Food has been suggested to be the main source of yersiniosis. It is critical for the researchers to be able to detect *Yersinia* or any other foodborne pathogen with increased sensitivity and specificity, as well as in real-time, in the case of a foodborne disease outbreak. Conventional detection methods are known to be labor intensive, time consuming, or expensive. On the other hand, more sensitive molecular-based detection methods like next generation sequencing, microarray, and many others are capable of providing faster results. DNA testing is now possible on a single molecule, and high-throughput analysis allows multiple detection reactions to be performed at once, thus allowing a range of characteristics to be rapidly and simultaneously determined. Despite better detection efficiencies, results derived using molecular biology methods can be affected by the various food matrixes. With the improvements in sample preparation, data analysis, and testing procedures, molecular detection techniques will likely continue to simplify and increase the speed of detection while simultaneously improving the sensitivity and specificity for tracking pathogens in food matrices.

1. Introduction

The genus *Yersinia* mainly includes animal pathogens, but animals can transmit disease to humans through direct or indirect contact [1]. Symptoms of illness can include diarrhea, vomiting, abdominal pain, and fever. There are three species within the genus *Yersinia* that are pathogenic for humans: *Yersinia enterocolitica, Yersinia pseudotuberculosis*, and *Yersinia pestis*. All these species have evolved with different clinical symptoms. *Y. enterocolitica* infections have been observed all over the world, but appear to be more common in Europe, especially in some Scandinavian regions, with much lower rates in the United States [2]. Food has often been suggested to be the main source of yersiniosis. Enteropathogenic *Yersinia*, *Y. enterocolitica,* and *Y. pseudotuberculosis,* entering the human body in contaminated food invade the M cells of the Peyer's patches [3]. The process and its effect on the host cell are driven by a large array of virulence factors that are deployed under genetic and environmental regulation. *Y. enterocolitica* can be categorized by biotype. Biotype 1A strain is considered as nonpathogenic, while 1B strain is considered as high-pathogenic, and biotypes 2, 3, 4, and 5 strains are considered as low-to-moderate pathogenic. The pathogenic phenotype can be differentiated due to the virulence-associated genes identified in these strains.

In the event of foodborne disease outbreaks, rapid identification of foodborne pathogens rely on the speed and simplicity of the detection method, which are critical for early detection and quick response [4]. The new advancement of high-throughput OMICS technologies provides scientists with the means to identify the agent and attribute it to a specific source of pathogenic *Yersinia* in food systems [5].

2. Current Advances in Detection Methods

One of the most challenging issues in food safety is the detection of foodborne pathogens. Since the infectious dose of many pathogens is as low as a few cells or particles [6], the sensitivity of the diagnostic tool becomes essential. In fact, the detection of pathogens in nonprocessed or minimally processed foods is not easy. Such foods are not sterile; the native microflora in such foods can mask the presence of a pathogen by interfering with isolation [7]. Thus, more sensitive and reliable detection methods have been developed in accordance with the advancement of molecular and biochemical technologies.

Isolation of *Y. enterocolitica* from clinical, food, and environmental samples can be challenging primarily due to the difficulty of growing *Y. enterocolitica in vitro* [8]. Traditional culture-dependent methods have several limitations, such as long incubation steps, lack of identification between species, and lack of discrimination between pathogenic and nonpathogenic strains [8, 9].

Numerous molecular techniques have emerged, that offer the advantage of speed along with specific and sensitive detection [10, 11]. Due to the relative simplicity, rapidity, reliability, and sensitivity, DNA-based detection technology plays an important role and provides detection methods in the form of next-generation sequencing [12], microarray [13], fluorescent in situ hybridization (FISH) [14], polymerase chain reaction (PCR) [15], molecular beacon technology [16], and many others. DNA testing is now possible on a single molecule, and high-throughput analysis allows thousands of detection reactions to be performed at once, thus allowing a range of characteristics to be rapidly and simultaneously determined. Some of the current molecular detection methods not only can be performed in the laboratory or clinical settings but also can be run at the observation site, such as on the farm or in the field, in the form of "all-in-one" kits [17, 18].

2.1. Genome Sequence. The release of the complete genome sequence of *Y. enterocolitica* strain 8081 provided important insights into the pathology of this bacterium [19]. There are 18 completed and over 160 incomplete *Yersinia* strains past and ongoing *Yersinia* genome sequencing projects (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi/) including *Y. pestis* strain CO92 [20] and *Y. pseudotuberculosis* strain IP 31758 at J. Craig Venter Institute/The Institute for Genomic Research [21]. These sequencing projects will enable the study of the evolution of the pathogenic changes in each species as they have adapted to new environmental surroundings. The information gathered from the genome sequences of the three major pathogenic *Yersinia* species will allow the development of a cross-species microarray for pathogenic *Yersinia* and will lead to invaluable insights into how the enteropathogens are adapted to their lifestyle.

Recently, Fuchs and coworkers took advantage of a whole-genome shotgun sequencing approach to assemble, annotate, and analyze the sequence of strain W22703 of *Y. enterocolitica* [22]. Their research study provided valuable information on the strategies utilized by *Y. enterocolitica* to cope with its environment. Wang et al. [23] sequenced the complete genome of *Y. enterocolitica* strain 3/O:9 and strain 8081 (1B/O:8); the comparison of the genome sequences of these two strains indicated that these two strains' different pathogenicity may have been a result of completely separate evolutionary events. Recent efforts by Batzilla et al. [24] to compare the complete genome of *Y. enterocolitica palearctic* serobiotype O:3/4 to the available genome of *Y. enterocolitica* ssp. *enterocolitica* 8081 O:8/1B indicated that gene loss and acquisition during evolution through mobile genetic elements could be the contributing factor to differentiate pathogenic bacteria from apathogenic bacteria of the same species. *Y. enterocolitica* is a heterogeneous bacterial species with a complex life cycle encompassing aquatic and biological environments. Further genome sequencing and analysis will help us to learn more about the evolution of *Y. enterocolitica* strains and provide the necessary information for the development of molecular-based detection methods for *Yersinia* in food systems.

Rouillard and Gulari developed a pangenomic oligonucletide microarray probe set database called OligoArrayDb [25]. OligoArrayDb was designed for most of the sequenced genomes that are not covered by commercial catalog arrays. Based on their algorithm of analysis, the *Y. enterocolitica* strain 8081 genome, a total of 4137 transcripts and containing 11821 oligonucletides, were chosen to represent the *Y. enterocolitica* strain 8081 transcriptome. Among these oloigonucleotides, 11251 are considered to be fully specific to their targets. This microarray probe set can be accessed through the website at http://berry.engin.umich.edu/oligoarraydb/index.html.

2.2. Microarray Analysis. The dominant application of microarrays has been in measuring gene expression in different biological conditions [26–28]. Other important microarray applications include comparative genomic hybridization [29], chromatin immunoprecipitation [30], mutation detection [31], genotyping [32], and array-mediated localized cell transfection [33, 34]. Microarray technology involves the placement of user-defined oligonucleotide probes in specific locations on a solid matrix such as glass or filters. The concept behind all microarrays is the precise placement of DNA fragments at high density on the solid support, so that they can act as molecular detectors. There are many variations of this method based on the solid matrix used and more importantly, the different types of DNA fragments on the array, including cDNA, oligonucleotides, and genomic fragments. Currently, there are three main types of microarrays: filter arrays [35], spotted glass slide arrays [36], and in situ synthesized oligonucluetide arrays [37] available for research purposes.

Following the hybridization of target DNA sequences to probes on the solid matrix, fluorescence-based detection can be used to monitor binding signal and be recorded. Along with the rapid development of microarray technologies, there has been an unprecedented amassing of data collected by academic institutes, as well as industrial organizations. Software applications can be used to conduct data analysis and greatly facilitate the data analysis

process. There are many open-source, public-domain, and commercial solutions for data storage, analysis, management, and exportation. Most of the applications are being updated frequently to keep current with the new demands from research. Several applications have been released that integrate data acquisition, processing, analysis, and exportation [25, 38]. The commercial GeneSifter (http://www.geospiza.com/Products/AnalysisEdition.shtml), the academic GenMAPP (http://genmapp.org/), and the open-source BASE (http://base.thep.lu.se/) aim to provide the functionalities for data analysis. Some software applications also provide comprehensive solutions for image analysis and data extraction. Most recent software applications for microarray data analysis are listed in Table 1.

Microarray methods provide an effective way of distinguishing between nonspecific and target product formation following PCR amplification of target DNA sequences from the samples. Amplification methods have been used previously in combination with microarray technology for the detection of *Y. pestis*. Huang et al. [39] were able to specifically detect *Y. pestis* from *Y. enterocolitica* and *Y. pseudotuberculosis* using a microarray method combined with PCR amplification. Myers and coworkers [40] developed a microarray chip combined with PCR amplification for detection and characterization of four virulence genes (*virF*, *ail*, *yst*, and *blaA*) in *Y. enterocolitica.* They were able to identify *Y. enterocolitica* from adulterated pasteurized whole milk using this approach. Ikeda et al. [41] were able to detect three foodborne bacteria: *Salmonella enterica* serovar Enteritidis, *Y. enterocolitica,* and *Bacillus cereus* in fresh vegetables using a DNA microarray method. Kim et al. [42] used comparative genomics to select 70-mer ologonucleotide probes specific for 11 major foodborne pathogens for use in microarray analysis. All of these studies have demonstrated that genome sequencing and DNA microarray analysis have a powerful application in detection of pathogenic *Yersinia* in food systems.

2.3. Immunoassay. Antibodies have been used for many years to type bacterial isolates serologically [43–45]. The development of the enzyme-linked immunosorbent assay (ELISA) introduced highly sensitive tests for specific targets with great reliability. Key advantages of ELISA are its ease of use, flexibility, and low cost. The highly specific nature of antibodies, especially monoclonal antibody (MAbs), and the simplicity and versatility of antigen-antibody reactions have facilitated the design of a variety of assays, and they comprise the largest group of molecular biological methods being used in foodborne pathogen detection [46–48].

Yersinia pestis is antigenically homogenous, but *Y. enterocolitica* and *Y. pseudotuberculosis* have multiple O and H antigens [49]. ELISA kits for detection of *Y. enterocolitica* are commercially available for the detection of the O antigen; for example, Mabs anti-O:3 and -O:9 can be purchased from LifeSpan BioSciences for research purposes.

Other methods for evaluating immunological binding events include fluorescence-based microscopy and surface plasma resonance. A commonly used field-portable immunoassay is the lateral flow disposable membrane technology. This technology is designed for threshold or qualitative testing. Advantages of this format include low-cost, portability, room-temperature stability and no need for specialized equipment and only minimal user training is required [50].

Multiplexing format immunoassays, suitable for the simultaneous evaluation of multiple targets in a sample, can be developed to increase the analytical productivity and drastically reduce analysis costs and sample and reagent consumption. For the low-multiplexing assay without automation, quantitative PCR, ELISA, or Western blotting allow multiple targets to be measured simultaneously and quantitatively. For the high multiplexing OMIC technologies, microarrays, SELDI, and LC/MS allow measurement of several hundred potential targets, but the output is essentially qualitative. There are two main multiplex immunoassay formats currently being applied widely in research: (1) protein attached microarrays [51, 52] and (2) bead-based microarrays [53, 54]. Magliulo et al. [55] developed a simple, multiplexed sandwich chemiluminescent enzyme immunoassay for the simultaneous detection of four of the major foodborne pathogens: *Escherichia coli* O157:H7, *Y. enterocolitica, Salmonella* Typhimurium, and *Listeria monocytogenes*. The accuracy and precision of this method were comparable to those achievable with the conventional culturing methodology yet detection was completed significantly faster than in traditional practices.

Protein microarray is a novel technology for quickly detecting and identifying proteins [56]. A protein detecting microarray comprises many different affinity reagents arrayed at high spatial density on a solid support. Each agent captures its target protein from a complex mixture, and the captured proteins are subsequently identified. For routine detection purposes, there is substantial benefit to be gained from using protein microarray technology. In principle, thousands of proteins can be spotted on a single slide, enabling one to interrogate simultaneously the presence of many different proteins with minimal sample consumption. Furthermore, hundreds of copies of an array can be manufactured, enabling the same proteins to be probed repeatedly with many different molecules from different samples. Rucker and coworkers have successfully developed antibody-based microarray techniques for the multiplexed detection of cholera toxin β-subunit, diphtheria toxin, anthrax lethal factor, and protective antigen, *Staphyloccus aureus* enterotoxin B, and tetanus toxin C fragment from spiked samples [57]. Li et al. used a protein microarray spotting with 149 *Y. pestis* proteins to profile antibody responses to a *Y. pestis* live vaccine [58]. With the continuing innovation for this technology, some limitations need to be addressed, as well. For protein detection microarrays, the cross-reactivity of affinity reagents need to be assessed and reduced. For a protein function microarray, the purity and integrity of the proteins need to be determined.

Immunoassays have an important role in the diagnosis and monitoring of diseases in routine-based pathological laboratories. However, immunoassay sensitivity and potential cross-reactivity should be carefully considered in comparing detection methods. Nucleic-acid-based technology

Table 1: Current software applications for microarray data analysis.

Software	Application	Provider	Platform	Web link
Array Designer	Primer design for microarray construction	Premier Biosoft International	Windows Linux	http://www.premierbiosoft.com/dnamicroarray/index.html
ArrayMiner	Analysis tool for microarray gene expression data	Optimal Design	Mac OS Windows	http://www.optimaldesign.com/ArrayMiner/ArrayMiner.htm
ArrayTrack	Database solution for managing, analyzing, and interpreting microarray gene expression data	National Center for Toxicological Research U.S. Food and Drug Administration	Web-based	http://www.fda.gov/ScienceResearch/BioinformaticsTools/Arraytrack/default.htm
ArrayVision	Automated analysis of macro- and microarrays	GE Healthcare	Windows	http://www.gelifesciences.com/aptrix/upp01077.nsf/Content/Products?OpenDocument&ParentId=957136
BAMarray	Detecting differentially expressed genes from microarray data using Bayesian analysis	Case Western Reserve University	Mac OS Windows Linux	http://www.bamarray.com/default.htm
BASE	Database solution for the massive amounts of data generated by microarray analysis	Lund University	Web-based	http://base.thep.lu.se/
Cluster	Perform a variety of types of cluster analysis and other types of processing on large microarray datasets	University of Tokyo	Mac OS Windows Linux/Unix	http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm
GenePattern	Gene expression analysis tools	Broad Institute, MIT	Web-based	http://www.broadinstitute.org/cancer/software/genepattern/desc/expression.html
GeneSifter	Tools for exploring the statistically significant interplay of the data with factors of biological relevance to understand the expression pattern in microarray data.	Geospiza Inc.	Web-based	http://www.geospiza.com/Products/AnalysisEdition.shtml
GenMAPP	Tools for visualizing data from gene expression experiments in the context of biological pathways.	Gladstone Institute, University of California at San Francisco	Windows	http://genmapp.org/
GenMaths XT	Analysis of high density microarrays and gene chips	Applied Maths	Windows	http://www.applied-maths.com/genemaths/genemaths.htm
Genowiz	A comprehensive multi platform software for microarray data analysis	Ocimum Biosolutions	Mac OS Windows Linux/Unix	http://www3.ocimumbio.com/data-analysis-insights/analytical-tools/genowiz/
Microarray tools	Including: a Comparative Genomic Hybridization (CGH) and expression microarray data analysis, data management and export system	J. Craig Venter Institute	Windows Linux/Unix	http://www.jcvi.org/cms/research/software/#c622/

Table 1: Continued.

Software	Application	Provider	Platform	Web link
Partek Genomics Suite	Statistical analysis and data mining tools to facilitate powerful and intuitive exploratory data analysis	Partek Incorporated	Windows Linux/Unix	http://www.partek.com/partekgs/
TreeArrange and Treeps	Software for displaying and manipulating hierarchical clustered data	University of Waterloo	Windows Linux/Unix	http://monod.uwaterloo.ca/downloads/treearrange/
waviCGH	For the analysis and visualization of array-CGH data	Spanish National Cancer Center, Bioinformatics Unit	Web-based	http://wavi.bioinfo.cnio.es/

may be a suitable alternative for a range of molecular targets traditionally detected by immunoassays [59].

2.4. Next-Generation Sequencing. DNA sequencing is one of the most important molecular tools in any life sciences field [12, 60]. Over the past 30 years, there has been more than a millionfold improvement in the rate of sequence generation with the progression from radio-labeled products using slab gels to fluorescent products and capillary electrophoresis to next-generation sequencing technologies [60]. According to Stratton, in the future, the cost of sequencing may drop greatly where, for example, the costs of sequencing whole cancer genomes can drop to US$1000. Routine sequencing in a clinical, diagnostic setting will then become feasible [60].

Next Generation Sequencing (NGS) technology has been adopted as a sequencing tool for quite some time [61–63]. This sequencing technology has the following features: massively paralleled sequencing without electrophoresis, samples need to be prepared and amplified, and extensive usage of computer resources. NGS can be categorized into (1) microelectrophoretic methods, (2) sequencing by hybridization, (3) real-time observation of single molecules, and (4) cyclic array sequencing [64].

There are significant differences between conventional sequencing technologies and NGS platforms in terms of sequencing chemistry, application, and cost [64, 65]. The comparison of major NGS technologies and conventional sequencing technologies is summarized in Table 2. The applications of conventional sequencing using the Sanger approach are suitable for small-scale sequencing within the kilobase to megabase range [66, 67]. The requirements of a Sanger sequencing approach include major costs such as robotic support of reagents, processing of multiple samples in either 96- or 384-well formats, and regular maintenance of capillary-based sequencers. NGS has fewer infrastructure requirements than the Sanger sequencing approach. Among the NGS platforms, there are important differences that may result in advantages with respect to specific applications (Table 2). Some applications may be more tolerant of short read lengths than others. The accuracy, as well as the specific error distributions of individual technologies, may also be relevant [68–71].

The diversity and advancement of NGS technology pose challenges for bioinformaticists to address, such as the issues of alignment, assembly, sequence scoring, data storage, and data release. Two major computational approaches are performed with NGS reads, assembly and alignment. The assembly approach is performed when no reference genome exists for the DNA sequenced, such as in the case of a genetically uncharacterized pathogen. Assembly algorithms take sequence reads, align overlapping sections, and generate longer length contigs, which serve as the scaffold for genome assembly, and subsequent alignments [72–74]. Alignment process is used to determine the best match between sequence reads and the reference sequence. To accommodate the large number of reads generated by NGS, a number of new alignment algorithms have been developed. These algorithms share the characteristic that alignment is performed in a multistep or heuristic approach in which the first phase consists of converting either the sequence reads or the reference sequence into an index of shorter length sequences, which are given read identifiers [75–77]. Postalignment, programs generate key information including the number of aligned reads, a list of sequence variants relative to the reference, and the percentage of reads containing the variant. A variety of software applications have been developed using these algorithms and are being widely utilized by researchers. Some of the popular tools are listed in Table 3.

Some of the key applications for NGS include (1) whole genome *de novo* sequencing and single nucleotide polymorphism (SNP) discovery [63, 68, 78], (2) mapping of structural rearrangements and transformation events [79], (3) expressed sequence tags (ESTs) or serial analysis of gene expression [80], (4) transcriptome assembly for gene discovery and transcription profiling [81], (5) large-scale analysis of DNA methylation [82], (6) genome-wide mapping of DNA-protein interactions [83], (7) confirmatory sequencing in gene cloning [84], and (8) genome-map-based cloning [85].

Cummings and coworkers [86] used the SOLiD system (Applied Biosystems, Calif) to conduct parallel microbial whole genome typing to detect strain-specific polymorphism in *Bacillus anthracis* and *Y. pestis*. Their research results

Table 2: Comparison of major next generation DNA sequencing technologies and conventional sequencing.

Platform	Application	Sequencing chemistry	Read length (bases)	Throughput per run (Gb)	Read per run (million)	Throughput per 24 hr (Gb)	Raw accuracy Range (%)	Cost Pe Mb ($)
ABI 3730	(1) Complement *de novo* assemblies for high-quality assembly of complex genomes; (2) Custom sequencing (3) Targeted resequencing for polymorphism discovery and genotyping	Sanger Dideoxy	800	0.00008	0.000096	0.00064	99.0 to 99.999	4000
ABI SOLID 5500	(1) Whole genome SNP discovery; (2) Transcriptome assembly and expression profiling; (3) Whole methylome resequencing	Sequencing by ligation	60×2	310	5167	45	99.0 to 99.9	0.05
Illumina HiSeq	(1) Whole genome SNP discovery; (2) Transcriptome assembly and expression profiling; (3) Whole methylome resequencing; (4) Bacterial and megaplasmid *de novo* assembly	Sequencing by synthesis	100×2	600	6000	75	96.2 to 99.7	0.02
Life Technologies Ion Torrent	(1) Whole methylome resequencing; (2) Bacterial and megaplasmids *de novo* assembly; (3) Sequencing quality control; (4) Sequencing requirement lower complexity	pH meter	200	0.2	1	2.4	>99.0	0.5
Roche 454	(1) *De novo* assemblies of complex genomes; (2) Metagenomics; (3) Analysis of large structural variations	Pyrosequencing	600	0.8	1	0.5	96.0 to 97.0	8

suggested the possibility of using NGS technology during a forensic or epidemiological investigation facilitating high-resolution strain tracking. Morelli et al. [87] utilized both conventional sequencing and NGS technologies to identify patterns of global phylogenetic diversity through the comparison of 17 whole genomes of *Y. pestis* isolates from global sources. Chen et al. used NGS technology to obtain and compare sequencing data from 3 pathogenic and 8 nonpathogenic members of the *Yersinia* genus [88]. They identified 100 regions within the genome of *Y. enterocolitica* that represented potential candidates for the design of nucleotide sequence-based assays for detection of the pathogen.

NGS has fundamentally impacted various fields of biological research, including food safety. This technology can be transitioned into the clinical diagnostic area. Similar to the development of microarray technology, the challenges will shift from mastering this technology to the question

Table 3: Software applications for NGS analysis.

Software	Categories	Sequencing file format compatibility	Created by	Operating platform	Web link
ABySS	Assembly	FASTA FASTQ QSEQ SAM BAM	Jared Simpson et al. Michael Smith Genome Sciences Centre	Mac OS Linux POSIX	http://www.bcgsc.ca/platform/bioinfo/software/abyss/
Edena	Assembly	FASTQ	David Hernandez University of Geneva Hospitals	Windows Linux	http://www.genomic.ch/edena.php/
Exonerate	Alignment	FASTA	Guy Slater and Ewan Birney European Bioinformatics Institute	Windows Linux Unix	http://www.ebi.ac.uk/~guy/exonerate/
Maq	Alignment	FASTA FASTQ Illumina Bustard & Gerald Illumina ELAND	Heng Li	Windows Linx	http://maq.sourceforge.net/
Mosaik	Alignment	FASTA FASTQ Illumina Bustard & Gerald SRF	Michael Stromberg and Gabor Marth Boston College	Mac OS Windows Linux	http://code.google.com/p/mosaik-aligner/
Phrap/ Cross_match/ Swat	Alignment	FASTA	Phil Green, Brent Ewing and David Gordon University of Washington	Mac OS Windows Linux	http://www.phrap.org/phredphrapconsed.html
PyroBayes	Base Caller	SFF	Aaron Quinlan et al. Boston College	Linux	http://bioinformatics.bc.edu/marthlab/PyroBayes/
SHARCGS	Assembly	Illumina Bustard & Gerald Illumina ELAND	Juliane Dohm et al. Max Planck Institute	Linux	http://sharcgs.molgen.mpg.de/
SHRiMP	Alignment	FASTA FASTQ SAM Illumina Bustard & Gerald	Michael Brudno and Stephen Rumble University of Toronto	Mac OS Linux	http://compbio.cs.toronto.edu/shrimp/
SOAP	Alignment Burrows-Wheeler	Illumina Bustard & Gerald Illumina ELAND	Ruiqing Li et al. Beijing Genomics Institute	Unix	http://soap.genomics.org.cn/
SSAHA2	Alignment Smith-Waterman	FASTA FASTQ SAM Illumina Bustard & Gerald	The Wellcome Trust Sanger Institute	Mac OS Linux	http://www.sanger.ac.uk/resources/software/ssaha2/
SSAKE	Assembly	FASTA	Rene Warren et al. Michael Smith Genome Sciences Centre	Linux	http://www.bcgsc.ca/platform/bioinfo/software/ssake/

Table 3: Continued.

Software	Categories	Sequencing file format compatibility	Created by	Operating platform	Web link
VCAKE	Assembly k-mer extension	FASTA	William Jeck et al.	Mac OS Linux	http://vcake.sourceforge.net/
Velvet	Assembly	FASTA FASTQ Illumina Bustard & Gerald Illumina ELAND	Daniel Zerbino et al.	Mac OS Linux Cygwin	http://www.ebi.ac.uk/~zerbino/velvet/

of how best to extract meaningful biological or clinical information from the large amount of data generated by this technology.

3. Summary

Food has often been suggested to be the main source of yersiniosis. Current methods to detect foodborne pathogens rely traditionally on culture media to select and propagate viable cells in foods. However, the isolation rates of pathogenic *Y. enterocolitica* have been low, which may be due to the limited sensitivity of the culture methods. The new advancement of the current technologies will provide cheaper, more accurate, and faster methods to identify pathogenic *Yersinia* in food systems during a food-related pathogenic crisis.

Despite better detection efficiencies, results derived using molecular biology methods can be affected by the various food matrices, the presence of normal bacterial flora, and interferences by some of the food ingredients. It still remains a challenge to develop methods that are rapid, sensitive, and specific in detection of foodborne pathogens. With the improvements in sample preparation, data analysis, and testing procedures, molecular detection techniques will likely continue to simplify and increase the speed of detection while simultaneously improving the sensitivity and specificity for tracking pathogens in food matrices.

The molecular-based detection methods discussed, above all, have advantages and limitations. Even use of the same detection method such as real-time PCR approach, different target genes used for the assay can limit the detection sensitivity. The detection range can vary from single colony forming unit (CFU) per ml to 10^3 CFU/mL. Similarly, the lateral flow stripe requires a relatively high concentration of target organisms between 10^7 CFU/mL to 10^{10} CFU/mL. Due to the limitations of individual detection methods, the combination with other techniques should be used for verification to ensure adequate specificity and sensitivity of the detection results. Combining with other methods also enhances the performance of individual assays. Owing to the complex variables in food analysis, most molecular-based methods for detecting foodborne pathogens are used for screening purposes, where the positive results need to be confirmed by cultural methods.

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Review Article

Behavior of *Yersinia enterocolitica* in Foods

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Yersinia enterocolitica are ubiquitous, being isolated frequently from soil, water, animals, and a variety of foods. They comprise a biochemically heterogeneous group that can survive and grow at refrigeration temperatures. The ability to propagate at refrigeration temperatures is of considerable significance in food hygiene. Virulent strains of *Yersinia* invade mammalian cells such as HeLa cells in tissue culture. Two chromosomal genes, inv and ail, were identified for cell invasion of mammalian. The pathogen can cause diarrhoea, appendicitis and post-infection arthritis may occur in a small proportion of cases. The most common transmission route of pathogenic *Y. enterocolitica* is thought to be fecal-oral via contaminated food. Direct person-to-person contact is rare. Occasionally, pathogenic *Y. enterocolitica* has been detected in vegetables and environmental water; thus, vegetables and untreated water are also potential sources of human yersiniosis. However, the isolation rates of pathogenic *Y. enterocolitica* have been low, which may be due to the limited sensitivity of the detection methods. To identify other possible transmission vehicles, different food items should be studied more extensively. Many factors related to the epidemiology of *Y. enterocolitica*, such as sources, transmission routes, and predominating genotypes remain obscure because of the low sensitivity of detection methods.

1. Introduction

Yersinia enterocolitica is a psychotropic zoonotic pathogen which causes acute gastroenteritis [1] and occasionally more serious disease in humans. In some countries it rivals *Salmonella* as a foodborne pathogen, and, because it can grow at refrigeration temperature [2], it is an increasing concern in terms of food safety. Infection with *Y. enterocolitica* can cause a variety of symptoms depending on the age of the person infected. Infection with *Y. enterocolitica* occurs most often in young children under 5 years old [3]. Most cases of yersiniosis occur sporadically in children [4]. The predominant symptoms in humans, particularly in young children, are fever, abdominal pain, and diarrhea, which is often bloody [5]. In older children and adults, the consequences of yersiniosis are severe and include acute infections, pseudoappendicitis, and extraintestinal long-term sequelae such as reactive arthritis and erythema nodosum [6, 7]. Secondary immunological sequelae, such as reactive arthritis, are not uncommon, especially in HLA-B27-positive individuals.

Yersinia enterocolitica is thought to be a significant food-borne pathogen, even though pathogenic strains have seldom been isolated from foods. Pigs are assumed to be the main reservoir of pathogenic *Y. enterocolitica* because pig is so far the only animal species from which pathogenic strains have frequently been isolated [8]. Several domestic animals like dogs, cats, cows, sheep, and horses and several wild [9] animals like rodents (mainly mice), monkeys, deer, and foxes have also been incriminated as potential reservoirs [10].

The geographical distribution of *Y. enterocolitica* is diverse. *Y. enterocolitica* has more than 50 distinct serotypes

(on the basis of antigenic variations in cell wall lipopolysaccharide), and few of them are pathogenic. O:8 is the primary infectious serotype in the USA followed by O:3, O:5,27, O:13a, 13b, O:20, O:9, and so forth, [11, 12]. Serotype O:3 is the most frequently isolated type in humans in Europe [3]. In China, serotype O:3 is primarily found in infections followed by O:9, and O:8 [13]. Furthermore, various serotypes demonstrate geographical specificity; for example, the predominant serotype in Australia, Europe, and Canada is O:3 [14], O:8 in Japan [15] and O:9 in Scandinavia, The Netherlands [16].

The emergence of yersiniosis is probably also related to changes that have occurred in livestock farming, food technology, and the food industry. Of greatest importance are changes in the meat industry, where meat production has shifted from small-scale slaughterhouses, with limited distribution patterns, to large facilities that process thousands of pigs each day and distribute their products nationally and internationally. Farm sizes have increased, and animal husbandry methods have also become more intensive. While many modern slaughter techniques reduce the risk of meat contamination, opportunities for animal-to-animal transmission of the organism and for cross-contamination of carcasses and meat products exist on a scale that was not known a few decades ago. In addition, advances in packaging and refrigeration now allow industry and consumers to store foods for much longer periods, a significant factor with regard to a cold-adapted pathogen such as *Y. enterocolitica.* In studying raw pork, higher detection rates have been obtained by PCR targeting chromosomally encoded several virulence genes than by culture methods [3]. In some case-controlled studies, an increased risk of yersiniosis has been demonstrated when raw or undercooked pork was consumed [14]. Nevertheless, the epidemiology of *Y. enterocolitica* infections is complex and remains poorly understood [3].

2. *Yersinia enterocolitica* Infection

Although *Y. enterocolitica* is a frequent and important cause of human disease in temperate zones, *Y. enterocolitica* infections have also been sporadically reported in tropical areas like China [19] and Japan [15]. The organism has been isolated from many foods, but foodborne outbreaks are rare, and most infections are sporadic. There have been relatively few foodborne outbreaks attributed to *Y. enterocolitica* in developed countries, for example, Japan, and The Netherlands [15, 16], as well as in developing countries, for example, Bangladesh and Iraq [20, 21].

Y. enterocolitica can cause gastrointestinal symptoms ranging from mild self-limiting diarrhoea to acute mesenteric lymphadenitis, which can lead to appendicitis [3]. The clinical manifestations of the infection depend to some extent on the age and physical state of the patient, the presence of any underlying medical conditions, and the bioserotype of the organism. Gastroenteritis, caused by *Y. enterocolitica*, is the most frequent form of yersiniosis, typically affecting infants and young children under 5 years [5]. In older children and young adults, acute yersiniosis can present as pseudoappendicular syndrome, which is frequently confused with appendicitis. Sometimes extra-intestinal long-term sequelae, including reactive arthritis, erythema nodosum, uveitis, glomerulonephritis, and myocarditis have been reported. Postinfection manifestations are mainly seen in young adults [3]. Sepsis is a rare complication of *Y. enterocolitica* infection, except in patients who have a predisposing underlying disease or are in an iron-overloaded state. Sepsis can also occur during blood transfusion [22]. In most cases, the infection is self-limiting, and no antimicrobial therapy is needed. However, in severe cases, antimicrobials may be useful. Antimicrobial resistance among human *Y. enterocolitica* strains has shown to be low, but multiresistant strains have also been reported [3], and, thus, antimicrobial therapy should always be based on the results of sensitivity tests.

Yersinia enterocolitica has evolved into an apparently heterogeneous collection of organisms encompassing six biotypes differentiated by physiochemical and biochemical tests (1A, 1B, 2, 3, 4, and 5) (Table 1) and more than 50 serotypes differentiated by antigenic variation in cell wall lipopolysaccharide. Of the six biotypes, biotype 1A is the most heterogeneous and encompasses a wide range of serotypes (Table 2), of which serotypes O:5, O:6,30, O:6,31, O:7,8, O:10, as well as O-nontypable strains are isolated most often [17]. The virulence of the pathogenic biotypes, namely, 1B and 2–5, is attributed to the presence of a highly conserved 70-kb virulence plasmid, termed pYV/pCD and certain chromosomal genes [23]. The biotype 1A strains of *Y. enterocolitica*, on the other hand, have been reported to lack pYV plasmid which encodes virulence factors including *Yersinia* adhesin A (YadA) and Ysc-Yop type III secretion system (TTSS) as well as chromosomally borne virulence genes including *ail*, *myfA*, *ystA*, *ysa*, and the high pathogenicity island- (HPI-) associated iron acquisition system [24].

Y. enterocolitica infection is typically initiated by ingestion of contaminated food or water. *Yersinia enterocolitica* (Figure 1) usually causes a diarrhoeal disease, whereas *Y. pseudotuberculosis* causes mild enteric symptoms that may be followed by mesenteric lymphadenitis and sometimes systemic diffusion. Yersiniae cross the intestinal epithelium primarily through the FAE, in the Peyer's patches of the ileum [25]. Invasin (Inv), a 103 kDa outer membrane protein of *Y. pseudotuberculosis* binds b1 integrins that are also expressed apically on M cells. Inv-negative mutants still adhere to and invade M cells, but at a much lower level than the wild-type strain, and their colonisation potential for Peyer's patches is considerably reduced [26].

Other *Yersinia* surface proteins such as Ail, PsaA, and YadA may account for residual invasion of inv mutants [28]. Once the dome is reached, yersiniae survive attack by resident macrophages by expressing an antiphagocytic strategy caused by the injection, through a plasmid-encoded type III secreton, of three protein effectors, YopH, T, and E, which disrupt cytoskeletal assembly [29]. YopH, a tyrosine phosphatase, dephosphorylates paxillin, p130cas, and FAK that are involved in the assembly of cytoskeletal complexes required for phagocytosis [30]. YopT provokes the depolymerisation of actin filaments by inducing redistribution of

Table 1: Biochemical tests used to biogroup *Y. enterocolitica* strains.

Test	Reaction for biotype[a]					
	1A	1B	2	3	4	5
Lipase activity	+	+	−	−	−	−
Salicin (acid production in 24 h)	+	−	−	−	−	−
Esculin hydrolysis (24 h)	+/−	−	−	−	−	−
Xylose (acid production)	+	+	+	+	−	V
Trehalose (acid production)	+	+	+	+	+	−
Indole production	+	+	V	−	−	−
Ornithine decarboxylase	+	+	+	+	+	+
Voges-Proskauer Test	+	+	+	+	+	+
Pyrazinamidase activity	+	−	−	−	−	−
Sorbose (acid production)	+	+	+	+	+	−
Inositol (acid production)	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	−

[a] Positive, negative; /: delayed positive; V: variable.

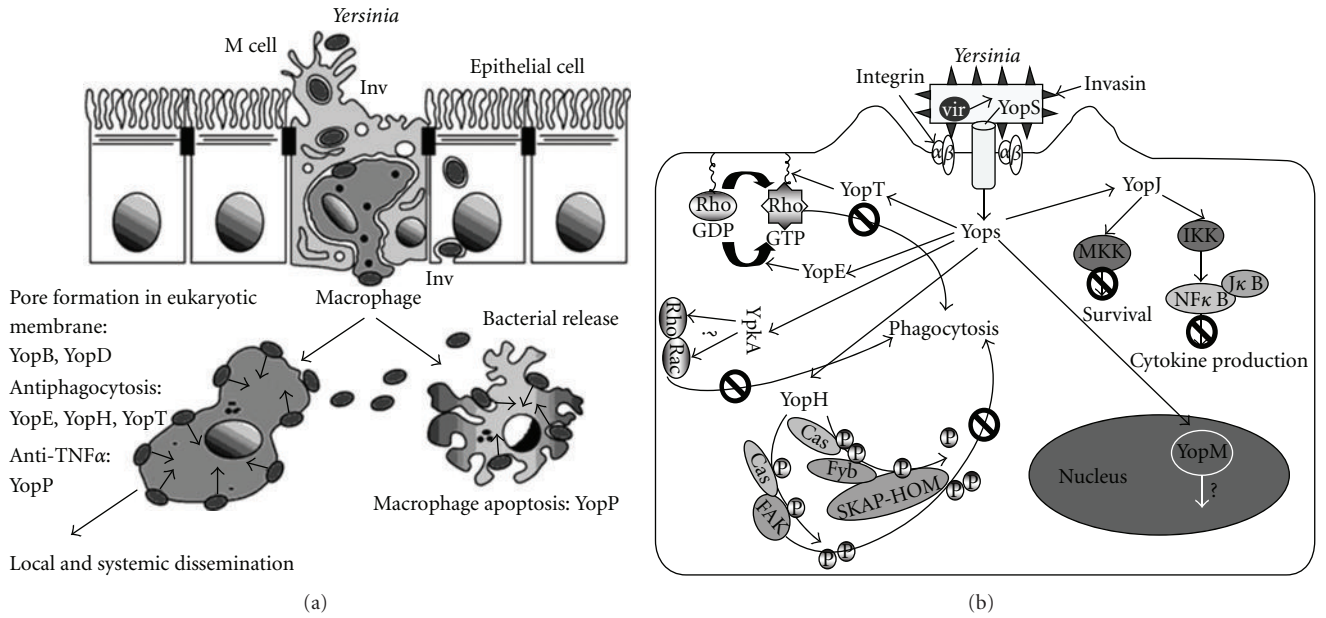

Figure 1: Physiopathological infection of *Yersinia* (adopted from [27]).

the RhoA GTPase [31]. YopE expresses a GAP function that inhibits the small GTPases of the Rho family involved in phagocytosis [32]. Yersiniae, therefore, remain essentially extracellular in infected Peyer's patches and mesenteric lymph nodes. This allows their extracellular survival and possible Inv-mediated entry into epithelial cells [27].

Y. enterocolitica strains belonging to certain few bioserotypes can cause human disease. Most strains associated with yersiniosis belong to the following bioserotypes: 1B/O:8; 2/O:5,27; 2/O:9; 3/O:3; 4/O:3. These bioserotypes have been shown to have different geographical distributions. Strains largely responsible for human yersiniosis in Europe, Japan, Canada, and the USA belong to the bioserotype 4/O:3 [33]. Strains of five biotypes (1B, 2, 3, 4, and 5) can carry the pYV, which is required for full expression of virulence, and several chromosomally encoded virulence determinants. Strains of biotype 1A lack the virulence-associated markers of pYV-bearing strains and are considered to be nonpathogenic. However, growing clinical, epidemiological, and experimental evidence suggests that some biotype 1A strains are virulent and can cause gastrointestinal disease [17]. Several studies have been conducted to investigate the distribution of different virulence genes (*ail*, *inv*, *yst*, *yadA*, *virF*, and *yopT*) among *Y. enterocolitica* strains by PCR [33]. Pathogenesis of *Y. enterocolitica* is mediated by virulence factors encoded on chromosomes and plasmids [34]. A correlation between biotypes and the presence of plasmid and chromosomal virulence genes has been found. However, plasmid-borne genes (*yadA*, *virF*, and *yopT*) have been detected with variable efficiency owing to heterogeneity

Table 2: Relatioship between biotype, O serotype and pYV carriage of *Y. enterocolitica* (adapted from [17]).

Biotype	Serotype(s)
lA	O:4; O:5; O:6,30; O6,31; O:7,8; O:7,13; O:10; O:14; O:16; O:21; O:22; O:25; O:37; O:41,42; O:46; O:47; O:57; NT[a]
1B	O:4,32[b]; O:8[b]; O:13a,13b; O:16; O:18[b]; O:20[b]; O:21[b]; O:25; O:41,42; NT
2	O:5,27[b]; O:9[b]; O:27
3	O:1,2,3[b]; O:3[b]; O:5,27[b]
4	O:3[b]
5	O:2,3[b]

[a]NT: not typable.
[b]Serotypes which include strains that carry pYV.

within the bacterial population for the presence of the virulence plasmid.

3. Epidemiology of *Y. enterocolitica*

Indirect evidence suggests that food, particularly pork, is an important link between the pig reservoir and human infections. In case-controlled studies, a correlation has been demonstrated between the consumption of raw or undercooked pork and the prevalence of yersiniosis [35, 36]. To identify reservoirs of infections, transmission vehicles, and associations between clinical cases, several DNA-based methods have been used to subtype *Y. enterocolitica* strains (Table 3). However, the high genetic similarity between *Y. enterocolitica* strains and the predominating genotypes among the strains have limited the benefit of these methods in epidemiological studies. Thus, many factors related to the epidemiology of *Y. enterocolitic*a, such as sources and transmission routes of yersiniosis, remain obscure.

4. Reservoirs

The evidence is not yet complete as to whether humans serve as reservoirs of *Y. enterocolitica*. It is isolated from low percentage of asymptomatic humans. However, it appears that the animal kingdom is a significant reservoir. Some members of the animal kingdom harbor unique serotypes of *Y. enterocolitica* which have not been implicated in human infections. Animals have long been suspected of being reservoirs for *Y. enterocolitica* and, hence, sources of human infections [11]. Numerous studies have been carried out to isolate *Y. enterocolitica* strains from a variety of animals. However, most of the strains isolated from animal sources differ both biochemically and serologically from strains isolated from humans with yersiniosis [10]. The pigs have been implicated as a major reservoir of *Y. enterocolitica* serotypes involved in human infections although a definite connection between the isolation of *Y. enterocolitica* from the pigs and human illness remains to be established. The incidence of *Y. enterocolitica* in pigs varies not only from country to country but also within a country. *Y. enterocolitica* strains that belong to bioserotypes associated with human disease have frequently been isolated from tonsils, tongues, and faecal samples of slaughtered pigs [14]. The rate of isolation of *Y. enterocolitica* from tonsils and tongues of pigs is generally greater than the rate of isolation from feces or fecal materials. In several countries, *Y. enterocolitica* of bioserotype 4/O:3 has been shown to be the predominant bioserotype in asymptomatic pigs. *Y. enterocolitica* serotype O:3 has been almost exclusively isolated from pigs in some European countries, like Denmark, Belgium, Finland, Germany, Sweden, and Switzerland [8, 37–40]. A lower prevalence has been reported in Italy, Greece, and Poland (Table 4) [41–43]. Some investigators concluded that the O:3 strain is a normal inhabitant of the oral cavity of pigs and also involved in human infection.

Examination of the throat flora from pigs in Ontario for *Y. enterocolitica* found the incidence of serotype O:3 to vary from 20% for tonsils to 50% for throat swabs and 55% for tongues. In contrast, there were no isolations of serotype O:3 from throat swabs taken from pigs in the western provinces of Canada. This incidence of serotype O:3 in pigs correlates well with the human incidence of the same serotype which is 81% for all human isolations of *Y. enterocolitica* in the eastern provinces and 4% in the western provinces of Canada. The opposite relationship is true for serotype O:5,27. The majority of O:3 and O:5,27 were positive for autoagglutination, a test which has been associated with virulence. The results suggest that pigs are an important source of human infections with both O:3 and O:5,27 [44].

In Guangxi, Mainland China, *Y. enterocolitica* were isolated from 48.4% of the pigs with diarrhea, and most of the isolates were O:3 with two isolates belonged to serotype O:9 [45]. These two serotypes are considered to be pathogenic for humans.

In another study in China, *Y. enterocolitica* (1,295 strains) was isolated from diarrhea patients, livestock, poultry, wild animals, insect vectors, food, and the environment. They were studied for epidemiology distribution using bacterial biochemical metabolism tests, their virulence genes, and pulsed-field gel electrophoresis (PFGE) subtyping. The data showed that 416 of the 1,295 strains were pathogenic, where the pathogenic Chinese isolates were of serotypes O:3 and O:9. These two serotypes were found in livestock and poultry, with pigs serving as the major reservoir. The geographic distribution of pathogenic isolates was significantly different, where most of the strains were isolated from the cold northern areas, whereas some serotype O:3 strains were recovered from the warm southern areas. By the analysis of the data of the Ningxia Hui Autonomous Region, the phenomenon of "concentric circle distribution" was found around animal reservoirs and human habitation. The clustering of PFGE showed that the patterns of the pathogenic strains isolated from diarrhea patients were identical compared to those from the animals in the same area, thus, suggesting that the human infection originated from the animals [19].

In many years of surveillance in China for *Y. enterocolitica*, no pathogenic O:8 strains have been found where the isolated O:8 serotypes lacked the major virulence genes, and,

Table 3: Methods for molecular typing of *Yersinia enterocolitica* isolates.

Typing method*	Typeability	Reproducibility	Discriminatory power	Use	Interpretation
REAP	Variable	Good	Poor	Easy	Easy
REAC	Excellent	Moderate	Moderate	Easy	Difficult
Ribotyping	Excellent	Excellent	Variable	Moderate	Easy
PFGE	Excellent	Excellent	Good	Moderate	Easy
PCR	Excellent	Moderate	Variable	Easy	Moderate
AFLP	Excellent	Good	Good	Moderate	Moderate
DNA sequencing	Excellent	Excellent	Good	Difficult	Moderate

Modified from viridi and Sachdeva [18].
*REAP: restriction endonuclease analysis of plasmid; REAC: restriction endonuclease analysis of chromosome; PFGE: pulsed-field gel electrophoresis; AFLP: amplified frgment length polymorphism.

Table 4: Annual incidence of disease caused by foodborne bacterial agents in different countries.

Country	Year	Cases	Incidence (per 100 000 population)
Australia	2000	73	0.6
Austria	1998	94	1.2
Belgium	2000	507	5
Denmark	2001	286	5.3
Finland	2001	728	14
Greece	1998	10	0.1
Japan	2001	4	<0.01
Norway	2001	123	2.8
Spain	1998	425	1.1
Sweden	2001	579	6.5
Switzerland	1998	51	0.7
United Kingdom	2000	27	0.05
United States	2002	164	0.44
New Zealand	2006	487	11.8

in contrast to the O:3 and O:9 strains, none of the O:8 isolates were from humans. These O:8 isolates lack *ail*, *ystA*, *yadA*, and *virF* genes but possess the *ystB* gene, and all belong to biotype 1A. These O:8 strains did not kill mice and could protect immunized mice against challenge with a pathogenic O:8 strain. Compared to the Chinese pathogenic O:3 and O:9 strains which have similar pulsed-field gel electrophoresis patterns, the 39 Chinese O:8 animal and food isolates were different from the pathogenic O:8 reference strains. This suggests the O:8 strains lacking virulence determinants may not disseminate rapidly in humans and are maintained in animal reservoirs, and, therefore, exhibit higher variance and divergence from the virulent type [13].

Sixteen different isolates of *Y. enterocolitica* were recovered from porcine tongues, including six O:8, four O:6,30, two O:3, and one each of O:13,7, O:18, and O:46 [46]. All the serotype O:8 isolates were virulent to mice, causing the death of adults after oral challenge [46].

In a cross-sectional study, individual pigs on eight swine operations were sampled for the presence of *Y. enterocolitica*. On each farm, both feces and oropharyngeal swabs were collected from pigs in five different production phases: gestating, farrowing, suckling, nursery, and finishing. A pig was considered positive if either sample tested positive. Of the 2,349 pigs sampled, 120 (5.1%) tested positive, and, of those, 51 were *ail* positive (42.5% of *Y. enterocolitica* isolates). On all farms, there was a trend of increasing prevalence as pigs mature. Less than 1% of suckling piglets tested positive for *Y. enterocolitica*. Only 1.4% (44.4% of which were *ail* positive) of nursery pigs tested positive, but 10.7% (48.1% of which were *ail* positive) of finishing pigs harbored *Y. enterocolitica*. Interestingly, gestating sows had the second highest prevalence of *Y. enterocolitica* at 9.1% (26.7% of which were *ail* positive), yet *Y. enterocolitica* was never detected from the farrowing sows [47].

Occasionally, pathogenic *Y. enterocolitica* strains, mostly of bioserotype 4/O:3, have been isolated from domestic animals like dogs, cows, horses, sheep, and cats [38]. Dogs excrete this organism in feces for several weeks after infection. *Y. enterocolitica* or related species were isolated from 50% of cows in Scotland, and the isolates varied in serotypes [48]. *Y. enterocolitica* strains of biotypes 2 and 3 and serotypes O:5,27 and O:9 have sporadically been isolated from slaughter pigs, cows, sheep, and goats; however, the reservoir of these bioserotypes is not clearly established [49–51]. Thus, pets may be one source of human infections because of their close contact with people, especially young children. *Y. enterocolitica* were isolated from wild animals

[52, 53] for example, from 16 of 495 small wild animals (mainly mice) and from 1 of 38 foxes [52], the isolated serotype were O:6, O:5A, O:4, and O:9. Wild rodents and pigs have been shown to be reservoirs for *Y. enterocolitica* O:8 strains in Japan [9]. Strains of very rare bioserotypes, such as bioserotype 5/O:2, 3, have been isolated from sheep, hares, and goats and bioserotype 3/O:1, 2a, 3 from chinchillas.

All environmental isolates, except one, had a *NotI* profile identical to that of an isolate recovered in pig feces from the same farm. This suggests that the environment represents a source of contamination of pigs by *Y. enterocolitica*. However, because the prevalence of pathogenic *Y. enterocolitica* in the environment was clearly lower than that in pigs, the pigs probably are the main source of pathogenic isolates on the farms. Several studies using different typing methods have been conducted to compare human strains with animal, mostly pig, strains. Most of the reports support the hypothesis that pigs are the main source of human *Y. enterocolitica* infections [33].

5. Contamination of Food and Environment

Food has often been suggested to be the main source of *Y. enterocolitica* infection, although pathogenic isolates have seldom been recovered from food samples. Raw pork products have been widely investigated because of the association between *Y. enterocolitica* and pigs. However, the isolation rates of pathogenic bioserotypes of *Y. enterocolitica* have been low in raw pork, except for edible pig offal, with the most common type isolated being bioserotype 4/O:3. The low isolation rates of pathogenic *Y. enterocolitica* in food samples may be due to limited sensitivity of culture methods [39]. The occurrence of pathogenic *Y. enterocolitica* in some foods has been estimated by different detection methods. In all of these studies, the prevalence was higher by PCR than by the culturing method.

Prevalence of *yadA*-positive *Y. enterocolitica* in food has been studied in Finland [39]. The highest detection rate was obtained from pig offal, including pig tongues (83%), livers (73%), hearts (71%), and kidneys (67%). The detection rate was higher in minced meat with the PCR method than with the culture method (Table 5). Thisted Lambertz and Danielsson-Tham [54] detected *ail*-positive *Y. enterocolitica* in 10% (9/91) of raw pork samples (loin, fillet, chop, ham, and minced meat) and in one of 27 ready-to-eat pork products. Surprisingly, Vishnubhatla et al. [55] found a high occurrence of *yst*-positive *Y. enterocolitica* in ground beef. In the same study, *yst*-positive *Y. enterocolitica* was also detected in tofu by real-time PCR. These PCR results indicate that the true rate of contamination of pathogenic *Y. enterocolitica* in pork and other processed meats and foods is underestimated using culture methods.

Y. enterocolitica has been isolated from raw milk in many countries, like Australia, Canada, Czechoslovakia, and USA. There were also a few reports on the isolation of this pathogen from pasteurized milk. It may be due to the malfunction in the pasteurization process leading to inadequate treatment or postprocess contamination, or it may be due to the contamination of heat-resistant strains of *Y. enterocolitica*. However, heat-resistant strains have not been reported.

Stern, 1982, reported that *Y. enterocolitica* could grow in whole milk at 3°C. Also the reduction of psychrotrophic bacteria in milk after pasteurization would enable a poor competitor and opportunistic pathogen such as *Y. enterocolitica* to grow better in pasteurized than in raw milk. So, the presence of this pathogen in pasteurized milk should be a cause for concern. *Y. enterocolitica* was isolated from 9.2% of cheese curd samples in Canada [60].

Y. enterocolitica are commonly detected in meat and poultry products. The level of this pathogen was found consistently in high numbers on vacuum-packed meats with

Table 5: Detection of pathogenic *Yersinia enterocolitica* in natural samples.

Sample	No. of samples	Reference
Clinical		
Pig tonsils	185	Fredriksson-Ahomaa et al. [37]
Pig tonsils	252	Boyapalle et al. [56]
Pig feces	255	Boyapalle et al. [56]
Mesenteric lymph nodes	257	Boyapalle et al. [56]
Food		
Pig tongues	51	Vishnubhatla et al. [55]
Minced pork	255	Fredriksson-Ahomaa and Korkeala [7]
Pig offal	34	
Chicken	43	Fredriksson-Ahomaa and Korkeala [7]
Fish	200	
Lettuce	101	
Pork[a]	300	Johannessen et al. [57]
Pig tongues	157	
Ground pork	100	Vishnubhatla et al. [55]
Ground beef	100	
Tofu	50	Vishnubhatla et al. [55]
Ground pork	350	Vishnubhatla et al. [55]
Chitterling	350	Boyapalle et al. [56]
Animal		
Cattle	46	Wang et al. [19]
Goats	160	Wang et al. [34]
Dogs	100	Wang et al. [34]
Swine	196	Wang et al. [34]
Poultry	68	Wang et al. [34]
Environmental		
Water	105	Sandery et al. [58]
Slaughterhouse	89	Fredriksson-Ahomaa et al. [59]
Flies	7	Wang et al. [34]

[a] Except pig tongues and offal.

a pH above 6 held at low temperature [60]. Growth of this pathogen is enhanced in cooked meats or at low temperature whereas competitive microorganisms are inactivated.

Prevalence of pathogenic *Y. enterocolitica* in different sources in Bavaria is presented. The highest isolation rate of pathogenic *Y. enterocolitica* (67%) was found in tonsils of slaughter pigs. No pathogenic strains were isolated from cattle, sheep, turkey, and horses. *ail*-positive *Y. enterocolitica* was detected in dogs (5%), cats (3%), and rodents (3%) by real-time PCR. Pathogenic *Y. enterocolitica* was isolated only from raw pork, especially from edible offal (51%). All pathogenic *Y. enterocolitica* isolates from nonhuman sources were belonging to bioserotype 4/O:3. All *Y. enterocolitica* 4/O:3 strains were susceptible to most of the tested antibacterial agents [61].

Strains of *Y. enterocolitica* have been isolated from oysters, mussels, shrimp, blue crab, fish, chicken salad, stewed mushrooms, cabbage, celery, and carrots [60].

No pathogenic *Y. enterocolitica* has been detected in fish and chicken samples in Finland; however, three (3%) lettuce samples were positive. In Korea, Lee et al. [62] isolated one *ail*-positive *Y. enterocolitica* strain of bioserotype 3/O:3 from 673 samples of ready-to-eat vegetables, which supports that vegetables can be a source of human infection. Furthermore, Sakai et al. [15] reported a foodborne outbreak of *Y. enterocolitica* O:8 in Japan where the same PFGE pattern was obtained from all patient and salad isolates. Recently, *Y. enterocolitica* 2/O:9 has been isolated from chicken eggshell surfaces in Argentina [63]. Using PFGE, *XbaI* patterns revealed a genomic heterogeneity among the strains, which suggests different contamination sources. Contamination of the egg surface might have occurred from contact with other *Y. enterocolitica*-contaminated animal products, such as pork, during collection on farms or during transportation or handling in retail shops.

In a case-controlled study, untreated drinking water has been reported to be a risk factor for sporadic *Y. enterocolitica* infections in Norway [36]. Drinking water has been relatively widely investigated and revealed to be a significant reservoir for nonpathogenic *Y. enterocolitica*. However, Sandery et al. [58] detected pathogenic *Y. enterocolitica* in 10% of environmental water, and Fālcao et al. [64] recently tested 67 *Y. enterocolitica* strains isolated in Brazil from untreated water for the presence of virulence genes. They found that all 38 strains of serotype O:5,27 possessed *inv*, *ail,* and *yst* genes, suggesting that water may be responsible for human infection with *Y. enterocolitica*. In Japan, the *Y. enterocolitica* O:8 strains have been isolated from stream water [9, 65]. Distribution of genotypes of *Y. enterocolitica* 4/O:3 strains in butcher shops in Munich has been studied with PFGE using NotI, ApaI, and XhoI enzymes [66]. Twelve genotypes were obtained among 33 isolates from 14 pork and two environmental samples, demonstrating that several different strains were distributed in butcher shops. The genotypes differed among butcher shops, possibly because raw material was purchased from different sources. In most shops, more than one genotype was found, indicating that the raw material was contaminated with different strains. These results show that pathogenic *Y. enterocolitica* can easily be transmitted from slaughterhouses via contaminated raw material to the retail level.

6. Possible Routes of Transmission

The most common transmission route of pathogenic *Y. enterocolitica* is thought to be fecal-oral via contaminated food. Direct person-to-person contact is rare. Lee et al. [67] reported *Y. enterocolitica* O:3 infections in infants who were probably exposed to infection by their careers. This may happen when basic hygiene and hand-washing habits are inadequate. In July 2006, person-to-person transmission was observed in a familial outbreak of *Y. enterocolitica* bioserotype 2/O:9 in Japan [68]. The possible source of this infection was an infected carrier suffered from diarrhea [68]. In addition, the outbreak of diarrheal disease due to *Yersinia enterocolitica* bioserotype 1/0:5 was reported in hospitalized patients, which was the indication of a nosocomial outbreak due to *Yersinia enterocolitica* [69]. Indirect person-to-person transmission has apparently occurred in several instances by transfusion of contaminated blood products [22]. One transmission link may be direct contact with pigs, a common risk for pig farmers and slaughterhouse workers. However, transmission of pathogenic *Y. enterocolitica* from pigs to humans has not yet been proven.

The main sources of human infection are assumed to be pork and pork products. Pathogenic *Y. enterocolitica* can be transmitted from slaughterhouses to meat-processing plants and then to retail level via contaminated pig carcasses and offal [66, 70]. Contaminated pork and offal are important transmission vehicles from retail shops to humans [70]. Cross-contamination of offal and pork will occur directly or indirectly via equipment, air and food handlers in slaughterhouses [59], retail shops [66], and residential kitchens. The detection rate of pathogenic *Y. enterocolitica* in raw pork products has been shown to be high. However, consumption of raw pork would play only a limited role in the development of yersiniosis as this is not a common habit in most developed countries. Nevertheless, in Germany, raw minced pork with pepper and onion is a delicacy that can be purchased in ready-to-eat form from butcher shops. Transmission probably more often occurs via cooked pork and other food products that have been undercooked or improperly handled.

Pet animals have also been suspected as being sources of human yersiniosis because of their close contact with humans, especially young children [34]. However, transmission from pets to humans has not yet been proven. Pathogenic *Y. enterocolitica* may be transmitted to humans indirectly from pork and offal via dogs and cats [38]. Transmission of *Y. enterocolitica* 4/O:3 to pets via contaminated pork has been studied using PFGE with NotI, ApaI, and XhoI enzymes. A total of 132 isolates, of which 16 were from cat and dog faeces and 116 from raw pork samples, were studied in Finland. The predominant genotype recovered from pig heart, liver, kidney, tongue, and ear samples was also found in the cat, whose diet consisted mostly of raw pig hearts and kidneys. The dog, which was fed raw minced pork, excreted the same genotype found in the minced meat. These results

show that raw pork should not be given to pets because pathogenic isolates can easily be transmitted from highly contaminated raw pork to pets. Dogs and cats may be an important transmission link of pathogenic *Y. enterocolitica* between pigs and young children [34].

7. Factors Influencing Survival and Growth

Y. enterocolitica is facultative organism and is able to multiply in both aerobic and anaerobic conditions.

7.1. Temperature. The ability of *Y. enterocolitica* to multiply at low temperatures is of considerable concern to food producers. The reported growth range is −2 to 42°C [71]. Optimum temperature is 28-29°C [72]. *Y. enterocolitica* can multiply in food such as meat and milk at temperatures approaching and even below 0°C [73]. It is important to recognize the rate at which *Y. enterocolitica* can multiply, which is considerably greater than that of *L. monocytogenes* [74]. Results showed that, in a food with a neutral pH stored at 5°C, *Y. enterocolitica* counts may increase from 10/mL to 2.8×10^7/mL in 5 days. Toxin production by this pathogen is affected by growth temperature and the composition of food items. Toxigenic *Y. enterocolitica* produced heat-stable enterotoxin in milk at 25°C but not at 4°C [75]. Strains which grew well at 4°C in milk did not produce significant amount of toxin to be detected by infant mouse assay [76]. Most *Y. enterocolitica* cells will be killed or injured when being stored during frozen storage at −20°C. When ground beef inoculated with *Y. enterocolitica* was stored at −20°C for 30 days, approximately 83% of the inoculated cells were destroyed and 24% of the survivors were sublethally injured [60].

7.2. pH. The minimum pH for growth has been reported between 4.2 and 4.4 [77], while in a medium in which the pH had been adjusted with HCl, growth occurred at pH 4.18 and 22°C [78]. The presence of organic acids will reduce the ability of *Y. enterocolitica* to multiply at low pH. Acetic acid is more inhibitory per gram molc than lactic and citric acid at a given pH [78]. Bactericidal activity order is acetic acid > lactic acid > citric acid > sulphuric acid. Bhaduri [79] performed an experiment by changing the pH of the food items at pH 4, 5, and 6. Number of viable cells decreased but 95% of the surviving cells retained the virulence plasmid with their virulence characteristics. However, plasmid-containing cells did not survive at pH 3 [79].

7.3. Water Activity. The minimum water activity at which growth occurred is 0.96. This bacterium was able to grow in 5% salt, but not in 7% salt. Stern et al. [77] tested four strains of *Y. enterocolitica* and reported that 0.945 Aw and 7% salt was bactericidal to all 4 strains tested, when incubated at 3°C, but at 25°C both bactericidal and bacteriostatic effects were observed. At 9% NaCl and 25°C, all 4 strains were killed. Bhaduri et al. [79] performed an experiment by changing the salt concentration of the food items to 0.5, 2, and 5%. Number of viable cells decreased, but 96% of the surviving cells retained the virulence plasmid with their virulence characteristics, indicating that there was no effect of NaCl (0.5, 2.0, and 5.0%) on pYV stability [79].

7.4. Preservatives/Disinfectants. The growth of *Y. entericolitica* is retarded by potassium sorbate up to 5000 ppm at pH 6.5 in a dose-dependent manner. At pH 5.5 concentrations above 1000 ppm virtually eliminate growth or cause inactivation depending on the dose. Sodium nitrite at a concentration of 150 ppm retarded growth on bologna. Treatments with ozone (1.4 and 1.9 ppm) and with ozonated water (1 min exposure) reduce pathogen loading [80]. Modified atmosphere packaging at 100% N_2 and CO_2/N_2 gas mixers inhibited the growth of *Y. entericolitica* at refrigeration temperatures.

8. Growth and Survival in Foods

The ability to propagate at refrigeration temperature in vacuum-packed foods with a prolonged shelf-life is of considerable significance in food hygiene. *Y. enterocolitica* may survive in frozen foods for long periods [81]. *Y. enterocolitica* is not able to grow at pH < 4.2 or >9.0 [82] or salt concentration greater than 7% (Aw < 0.945) [83]. *Y. enterocolitica* is not heat-resistant bacteria; with D value at 62.8°C for 15 enterotoxigenic and 6 nonenterotoxigenic cultures ranged from 0.7 to 17.8 sec. in sterile whole milk, the heat-treated cells were counted on trypticase soy agar with yeast extract [75]; it indicates that it does not survive pasteurization. The organism does not survive pasteurization or normal cooking, boiling, backing, and frying temperatures. Heat treatment of milk and meat products at 60°C for 1–3 min effectively inactivates *Y. enterocolitica* [73]. D values determined in scalding water were 96, 27, and 11 seconds at 58, 60, and 62°C, respectively. In another report [84], three raw milk isolates of *Y. enterocolitica* had D values at 62.8°C from 0.24 to 0.96 min in sterile whole milk. However, if the initial level of *Y. enterocolitica* is very high, complete destruction may not occur during pasteurization [60]. Sublethal injury of *Y. enterocolitica* may occur when the cells are treated at 47°C for 12–70 min [60].

A comparison of published and predicted generation times for *Y. enterocolitica* in raw pork at 7°C, 0.5% NaCl (w/v), and pH 5.5–6.5 shows GTs of 8.4–12.4 hours (published) and 8.15–5.05 hours (predicted). However, according to many reports, the ability of *Y. enterocolitica* to compete with other psychrotrophic organisms normally present in food may be poor [85]. In contrast, a number of studies have shown that *Y. enterocolitica* is able to multiply in foods kept under chill storage and might even compete successfully [86, 87]. The effect of lactic acid (concentration range 1.0 to 1.1% v/v within a pH range of 3.9 to 5.8 at 4°C) on growth of *Y. enterocolitica* O:9 is greater under anaerobic than aerobic conditions, although the bacterium has proved to be more tolerant of low-pH conditions under anaerobic atmosphere in the absence of lactic acid [88].

Pig carcasses are often held in chilling rooms for 2–4 days after slaughter prior to cutting. Prepackaged raw meat products may remain in retail chill cabinets for more than a week, depending on the product, packaging, package

atmosphere, and rate of turnover. Pathogenic strains of *Y. enterocolitica* might propagate considerably during the course of this relatively long storage period.

As a facultative organism, the growth of *Y. enterocolitica* is drastically affected by a gaseous atmosphere. Under anaerobic conditions, *Y. enterocolitica* is unable to grow in beef at pH 5.4–5.8, whereas growth occurs at pH 6.0 [89]. One hundred percent CO_2 is reported to inhibit the growth of *Y. enterocolitica* [89]. In the study of Gill and Reichel [71], *Y. enterocolitica* was inoculated into high beef DFD (dark-firm-dry) meat. Samples were packaged under vacuum or in oxygen-free CO_2 atmosphere maintained at atmospheric pressure after the meat had been saturated with gas and stored at −2, 0, 2, 5, or 10°C. In vacuum packs, *Y. enterocolitica* grew at all storage temperatures at rates similar or faster than those of the spoilage microflora. In CO_2 packs, the bacterium grew at both 5 and 10°C, but not at lower temperatures. Growth of *Y. enterocolitica* was nearly totally inhibited both at 4 and 10°C in a 60% CO_2/0.4% CO mixture, while the bacterial numbers in samples packed in high O_2 mixture (70% O_2/30% CO_2) increased from about 5×10^2 bacteria/g at day 0 to about 10^4 at day 5 at 4°C and to 10^5 at 10°C. Growth in chub packs (stuffed in plastic castings) was even higher [89].

The influence on *Y. enterocolitica* counts of a gradual increase of carbon dioxide concentrations (percentage by volume in air) during packaging and storage of ground pork meat artificially contaminated with this pathogen was evaluated. Ground meat was packaged under customary conditions using modified atmospheres with various carbon dioxide percentages (0, 30, 50, 70, and 100% CO_2 by volume; for atmospheres of less than 100% CO_2, the rest of the gas was O_2). The packs were stored at 2°C for 12 days. *Y. enterocolitica* counts were not significantly different ($P >$ or $=0.05$) in the ground pork packaged under the various CO_2-enriched atmospheres. The growth of *Y. enterocolitica* was nearly entirely inhibited in all tested modified atmospheres containing the protective CO_2. However, in ground pork packaged with 100% oxygen, there was a significant decrease ($P <$ or $=0.05$) for *Y. enterocolitica* from 4.30 log CFU/g (day 0) to 3.09 log CFU/g at the end of the storage time (day 12). The decrease was presumably due to the marked increase in aerobic plate count seen only in those packages stored under 100% O_2. Packaging with high CO_2 concentrations had significant inhibitory effect ($P <$ or $=0.05$) on the growth of mesophilic aerobic bacteria [90].

Mohammad and Draughon [91] investigated the growth characteristics of *Y. enterocolitica* strains in pasteurized milk at 4°C. Pasteurized milk was inoculated with 10 or 1000 cells/mL of *Y. enterocolitica*. *Y. enterocolitica* competed well with the background microflora and reached levels of log 5.0 to 7.0/mL after 7 days. However, a study by Stern et al. [77] indicated that while *Y. enterocolitica* has the capacity for growth in milk at refrigeration temperatures, it is a poor competitor with common spoilage organisms.

Some strains of *Y. enterocolitica* are even able to grow in water at low temperatures (4°C) [92]. In a study, autoclaved tap water (pH 6.5) was chlorinated according to conventional water treatment practices, resulting in a free residual level of approximately 0.05 mg/L after contact time of 30 min. A 3.0 log reduction for *Y. enterocolitica* and *E. coli* exposed to 0.2 mg/L Cl_2 was obtained in 20–180 and 20–25 sec, respectively, depending on the bacterial strain, plasmid content (*Y. enterocolitica* O:3 harbouring a 40–50 MDa virulence plasmid exhibits enhanced resistance to chlorine), and temperature [93].

Bansal et al. [94] performed an experiment to determine whether the presence of pYV plasmid affects the susceptibility of *Y. enterocolitica* to widely used antimicrobial agents like chlorine and heavy metals. According to them plasmid-bearing (pYV+) *Y. enterocolitica* was less susceptible to the antimicrobial action of chlorine and heavy metals compared with the isogenic plasmidless (pYV−) derivative. This difference was, however, observed only with bacteria cultured at 25°C but when cells cultured at 37°C were also found to be less susceptible to the antimicrobial action of these agents. These results indicate that the susceptibility of *Y. enterocolitica* to these agents was influenced both by the presence of the virulence plasmid and the temperature at which the cells were cultured [94].

Experiments were conducted to determine the effectiveness of oregano and nutmeg essential oils (EOs) on the growth and survival of *Y. enterocolitica* and *Listeria monocytogenes* in broth culture and in Iranian barbecued chicken. Ready-to-cook Iranian barbecued chicken was prepared according to the common practice with 1, 2, and 3 microL/g of oregano and nutmeg EOs. The test and control (without EOs) samples were inoculated with *Y. enterocolitica* to a final concentration of 6 to 7 log CFU/g and stored at 3, 8, and 20°C. Microorganisms were counted just before and at 24, 48, and 72 h after storage. However, the oregano EO had a greater effect on *Y. enterocolitica* (MIC = 0.16 microl/mL) than did the nutmeg EO (MIC = 0.25 microl/mL). In ready-to-cook Iranian barbecued chicken, the log CFU per gram of the bacteria after up to 72 h of incubation was not decreased significantly by various combinations of oregano and nutmeg EOs (1, 2, and 3 microl/g) and storage temperatures (3, 8, and 20°C) when compared with control samples (without EOs). Although examination of spices in culture media can yield accurate microbiological data, without complementary tests in foods, these data are of limited value for assessing food safety [95].

9. Conclusion

Yersinia enterocolitica is an important zoonotic pathogen that can cause yersiniosis in humans and animals. Pigs are assumed to be the main source of human yersiniosis, even though a definite connection between pathogenic *Y. enterocolitica* strains isolated from pigs and human infections has not been established. A close genetic relationship between pig and human strains of *Y. enterocolitica* has been demonstrated by several DNA-based methods. However, the high similarity between strains and the predominating genotypes within the bioserotype have limited the benefit of these detection methods in epidemiological studies. This method could provide a means of discriminating *Y. enterocolitica* strains found to be identical with other epidemiological tools.

There are considerable difficulties associated with isolating *Y. enterocolitica* from clinical, food, and environmental samples. Conventional culture-dependent methods have several limitations, such as low sensitivity, long incubation time, lack of identification between species, and lack of discrimination between pathogenic and nonpathogenic strains. Using PCR, pathogenic *Y. enterocolitica* can be detected in natural samples rapidly and with high specificity and sensitivity. Recently, several real-time PCR assays for qualitative detection of *Y. enterocolitica* in clinical, food, and environmental samples have been developed. However, to date, the PCR method has been used in only a few studies.

Prevalence of pathogenic *Y. enterocolitica* in pigs has been determined by PCR in some countries; however, epidemiological data about other possible animal reservoirs and from many countries are still missing.

Food has often been suggested to be the main source of yersiniosis, although pathogenic strains have seldom been isolated from food samples. Raw pork products have been widely investigated because of the association between *Y. enterocolitica* and pigs. However, the isolation rates of pathogenic *Y. enterocolitica* have been low, which may be due to the limited sensitivity of the detection methods. Occasionally, pathogenic *Y. enterocolitica* has been detected in vegetables and environmental water; thus, vegetables and untreated water are also potential sources of human yersiniosis. To identify other possible transmission vehicles, different food items should be studied more extensively.

Using genotyping, only a few animal reservoirs of *Y. enterocolitica* infections have been identified. The primary source of pathogenic *Y. enterocolitica* is fattening pigs. A close genetic relationship between pig and human strains of *Y. enterocolitica* has been demonstrated by several DNA-based methods. Human pathogenic *Y. enterocolitica* strains share common genotypes with dog strains, indicating that dogs are a possible source of human yersiniosis. In Great Britain, sheep are suspected of being a potential reservoir of human yersiniosis. Similar AFLP patterns between human and sheep strains reinforce this assumption. Wild rodents have been shown to be an important reservoir of *Y. enterocolitica* O:8 strains in Japan. Indistinguishable genotypes have been found among strains isolated from humans and wild rodents. Tonsils of fattening pigs are an important contamination source in slaughterhouses. *Yersinia*-positive tonsils will easily contaminate the carcass, the offal, and the environment during the slaughtering process. Using PFGE, *Yersinia*-contaminated pork and edible pig offal has proven to be important transmission vehicles of pathogenic *Y. enterocolitica* from the slaughterhouse to the retail level and further to humans. Indirect transmission of pathogenic *Y. enterocolitica* from pets to humans may occur via contaminated pork and offal. Indistinguishable genotypes have been found among strains isolated from humans and environmental water, indicating that untreated water is a possible infection source for human yersiniosis. However, many factors related to the epidemiology of *Y. enterocolitica*, such as sources and transmission routes, remain obscure because of the low sensitivity of detection methods and the predominating genotypes among *Y. enterocolitica* strains.

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Review Article

Yersinia enterocolitica: Epidemiological Studies and Outbreaks

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Yersinia enterocolitica is the most common bacteriological cause of gastrointestinal disease in many developed and developing countries. Although contaminated food is the main source of human infection due to *Y. enterocolitica*, animal reservoir and contaminated environment are also considered as other possible infection sources for human in epidemiological studies. Molecular based epidemiological studies are found to be more efficient in investigating the occurrence of human pathogenic *Y. enterocolitica* in natural samples, in addition to conventional culture based studies.

1. Introduction

Foodborne diseases are a widespread and growing public health problem in developed and developing countries [1]. Amongst those, yersiniosis due to infection with the bacterium *Yersinia enterocolitica* is the frequently reported zoonotic gastrointestinal disease after campylobacteriosis and salmonellosis in many developed countries, especially in temperate zones [2]. Within developed countries, incidences of yersiniosis and foodborne outbreaks are appeared to be lower in the United States than many European countries [3–5]. In European countries, numbers of reported cases of human in England and Wales are lower than those in other European countries where fewer than 0.1 cases of yersiniosis per 100,000 individuals were reported in the United Kingdom in 2005, in contrast to 12.2 in Finland and 6.8 in Germany [6]. On the other hand, the high prevalence of gastrointestinal illness including fatal cases due to yersiniosis is also observed in many developing countries like Bangladesh [7], Iraq [8], Iran [9], and Nigeria [10], which indicates major underlying food safety problems in low- and middle-income countries. Worldwide, infection with *Y. enterocolitica* occurs most often in infants and young children with common symptoms like fever, abdominal pain, and diarrhea, which is often bloody. Older children and young adults are not out of risk. The predominant symptoms within these age groups are right-sided abdominal pain and fever, sometimes confused with appendicitis. Occasionally, the *Y. enterocolitica* associated complications such as skin rash, joint pains, or spread of bacteria to the bloodstream can also occur.

Although *Y. enterocolitica* is a ubiquitous microorganism, the majority of isolates recovered from asymptomatic carriers, infected animals, contaminated food, untreated water, and contaminated environmental samples are nonpathogenic having no clinical importance [11]. At the same time, the epidemiology of *Y. enterocolitica* infections is complex and remains poorly understood because most sporadically occurred cases of yersiniosis are reported without an apparent source [3, 12–14]. However, most pathogenic *Y. enterocolitica* strains associated with human yersiniosis belong to bioserotypes 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3. Within these reported strains, fully pathogenic strains carry an approximately 70 kb plasmid termed pYV (plasmid

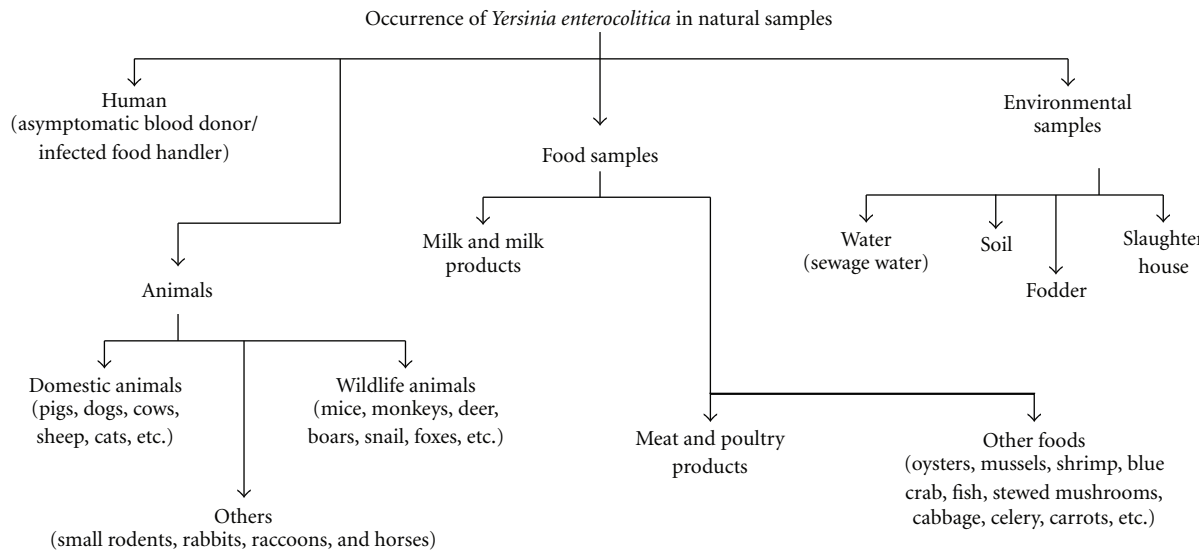

Figure 1: Occurrence of *Y. enterocolitica* in natural samples.

for *Yersinia* virulence) [15] that encodes various virulence genes (*tccC*, *yadA*, *virF*, *ysa)* with traditional chromosomal virulence genes (*inv*, *ail*, *yst*) whereas other pathogenic strains, having no pYV plasmid, produce a thermostable enterotoxin (*yst*A) [16–18]. These virulence genes located in chromosome or plasmid of pathogenic *Y. enterocolitica* has been widely used to identify pathogenic strains in epidemiological studies for example, chromosomal *ail* gene [19, 20].

2. Epidemiological Studies and Outbreaks

Many factors related to the epidemiology of *Y. enterocolitica*, such as human and nonhuman sources, and contamination routes in foods remain obscure in developing countries and tropical regions of developed countries. Additionally, epidemiological data on the prevalence of pathogenic *Y. enterocolitica* in animals in developed countries are missing as the reporting of this pathogen in animals is not mandatory in most European countries [26].

2.1. Animal Reservoirs Involved in Zoonosis. Animals have long been suspected of being significant reservoirs for *Y. enterocolitica* and, therefore, sources of human infections [3]. Numerous studies have been carried out to isolate *Y. enterocolitica* strains from a variety of animals (Figure 1) [56]. Interestingly, most of the strains isolated from the animal kingdom carry unique serotypes of *Y. enterocolitica* compared to the strains isolated from humans with yersiniosis.

Pigs have been shown to be a major reservoir of pathogenic *Y. enterocolitica* involved in human infections, particularly for strains of bioserotype 4/O:3 which has been almost exclusively isolated in European countries like Denmark, Italy, Belgium, Spain, and Sweden [24, 64]. The rate of isolation of *Y. enterocolitica* including bioserotype 4/O:3 from tonsils and tongues of pigs is generally greater than the rate of isolation from cecal or fecal materials [20].

Occasionally, pathogenic *Y. enterocolitica* strains, mostly of bioserotype 4/O:3, have also been isolated from dogs and cats [82]. Although pigs are the primary source of human infection with *Y. enterocolitica* throughout world, these pets may also be a potential source of human infection with pathogenic *Y. enterocolitica* because of their intimate contact with people, especially young children [28].

In addition with mostly isolated bioserotype 4/O:3, *Y. enterocolitica* strains of biotypes 2 and 3 and serotypes O:5,27, O:8, and O:9 have also been isolated from slaughter pigs, cows, sheep, and goats; however, the reservoir of these bioserotypes is not clearly established [81, 83–85]. In above cases, contamination of pluck sets (tongue, tonsils, and trachea hanging together with thoracic organs such as lungs, liver, and heart) and carcasses with enteropathogenic *Yersinia* from tonsils and feces may occur during the slaughtering stage [5, 82, 86–88]. On the other hand, strains of very rare bioserotypes, such as bioserotype 5/O:2,3, have been isolated from sheep, hares, and goats and bioserotype 3/O:1,2a,3 from chinchillas (small rodent). Thus, the patterns of the pathogenic strains isolated from humans with yersiniosis compared to those from the animals suggest that the human infection due to *Y. enterocolitica* originated from the animals.

2.2. Contaminated Food Involved in Infections. Food has been proposed to be the main source of intestinal yersiniosis although pathogenic isolates have seldom been recovered from food samples [105]. The low recovery rates of pathogenic *Y. enterocolitica* in food samples may be due to limited sensitivity of culture methods [11]. However, *Y. enterocolitica* has been isolated from milk and milk products, egg products, raw meats (beef, pork, and lamb) and poultry, vegetables, and miscellaneous prepared food products. The occurrence of pathogenic *Y. enterocolitica* in natural sample

Table 1: Detection of pathogenic *Y. enterocolitica* in natural samples with PCR and culture methods.

Sample	No. of samples	No. of culture^{+ve} samples[a] (%)		No. of PCR^{+ve} samples (%)		References
Animal						
Pig tonsils	185	48	(26)	58	(31)	Fredriksson-Ahomaa et al. [21]
	252	0		90	(36)	Boyapalle et al. [22]
	24	15	(63)	18	(75)	Nesbakken et al. [23]
	829	411	(50)	0		Martínez et al. [24]
	630	278	(44)	0		Martínez et al. [25]
	212	72	(34)	186	(88)	Fredriksson-Ahomaa et al. [26]
Pig faeces	255	0		80	(31)	Boyapalle et al. [22]
	24	3	(13)	3	(13)	Nesbakken et al. [23]
	2793	114	(4)	345	(12)	Bhaduri et al. [27]
	150	3	(2)	0		Okwori et al. [10]
Mesenteric l. n.	257	0		103	(40)	Boyapalle et al. [22]
	24	1	(4)	2	(8)	Nesbakken et al. [23]
Submaxillary l. n.	24	1	(4)	3	(13)	Fredriksson-Ahomaa et al. [20]
Sheep feces	200	2	(1)	0		Okwori et al. [10]
Dog feces	448	0		6	(1)	Wang et al. [28]
Food[b]						
Pig tongues	15	7	(47)	10	(67)	Vishnubhatla et al. [29]
	99	79	(80)	82	(83)	Fredriksson-Ahomaa and Korkeala [11]
Pig offal[c]	110	38	(35)	77	(70)	Fredriksson-Ahomaa et al. [20]
Chitterlings	350	8	(2)	278	(79)	Boyapalle et al. [22]
Ground pork	350	0		133	(38)	Fredriksson-Ahomaa et al. [20]
	100	32	(32)	47	(47)	Vishnubhatla et al. [29]
Ground beef	100	23	(23)	31	(31)	Fredriksson-Ahomaa et al. [20]
Minced pork	255	4	(2)	63	(25)	Fredriksson-Ahomaa and Korkeala [11]
Pork[d]	300	6	(2)	50	(17)	Johannessen et al. [30]
	91	6	(7)	9	(10)	Lambertz & Danielsson-Tham [31]
	62	0		20	(32)	Grahek-Ogden et al. [32]
Chicken	43	0		0		Fredriksson-Ahomaa et al. [11]
Fish	150	0		0		Okwori et al. [10]
Heated soup	100	3	(3)			Okwori et al. [10]
Cow milk	250	3	(1)			Okwori et al. [10]
Lettuce	250	0		3	(3)	Okwori et al. [10]
Tofu	50	0		6	(12)	Vishnubhatla et al. [29]
Vegetables	27	1	(4)	4	(15)	Cocolin & Comi [33]
Salad	42	16	(38)	16	(38)	Sakai et al. [34]
Environment						
Water	105	1	(1)	11	(10)	Sandery et al. [35]
Slaughterhouse/ Farm	89	5	(6)	12	(13)	Fredriksson-Ahomaa et al. [36]
	46	44	(96)	0		Martínez et al. [24]
	45	31	(61)	0		Martínez et al. [25]

[a] Pathogenicity of isolates confirmed, [b] all meat samples are raw, [c] liver, heart, kidney, [d] except pig offal & tongues, and $^{+ve}$ positive.

including foods has been estimated by both culture- and molecular-based methods (Table 1, Figures 2 and 3).

2.2.1. Contaminated Meat and Poultry Products Correlated with yersiniosis. Indirect evidence considering food, particularly pork and pork products, indicates that there is an important link between consumption of raw, undercooked, or improperly handled pork product and human *Y. enterocolitica* infections [20]. This positive correlation between the consumption of raw or undercooked pork and the prevalence of yersiniosis has been demonstrated in case-control studies [32, 64, 106–109]. Using molecular techniques, *ail*-positive *Y. enterocolitica* strains were detected in raw pork samples (loin, fillet, chop, ham, and minced meat) and in ready-to-eat pork products [31]. However, the isolation rates of pathogenic bioserotypes of *Y. enterocolitica* have been low in raw pork, except for in edible pig offal, with the most common type isolated being bioserotype 4/O:3 (Table 2). In

Table 2: Detection of pathogenic *Y. enterocolitica* in pork products by culture methods (partially adapted from Fredriksson-and Korkeala [11]).

Sample	No. of samples	No. of samples positive for				Country of origin of sample	Reference
		O:3	O:5,27	O:8	O:9		
Tongue	302	165			3	Belgium	Wauters [37]
	37	11				Canada	Schiemann [38]
	31	2		6		USA	Doyle et al. [39]
	47	26				Norway	Nesbakken [40]
	50	20				Japan	Shiozawa et al. [41]
	125	8				Spain	Ferrer et al. [42]
	29	28				Belgium	Wauters et al. [43]
	40	6			2	The Netherlands	de Boer and Nouws [44]
	55	14				Germany	Karib and Seeger [45]
	86	2				Italy	de Guisti et al. [46]
	99	79				Finland	Fredriksson-Ahomaa et al. [47]
	20	15				Germany	Fredriksson-Ahomaa et al. [48]
Tonsil	89	81			8	Belgium	Martínez et al. [24]
	137	136	1			Italy	Martínez et al. [24]
	185	185				Spain	Martínez et al. [24]
	212	69	6		1	Switzerland	Fredriksson-Ahomaa et al. [26]
Offal[a]	34	17				Finland	Fredriksson-Ahomaa et al. [36]
	16	5				Finland	Fredriksson-Ahomaa et al. [47]
	100	46				Germany	Fredriksson-Ahomaa et al. [48]
Pork[b]	91	1		1		Canada	Schiemann [38]
	127	1				Norway	Nesbakken et al. [49]
	70	22			3	Japan	Shiozawa et al. [41]
	267	6				Denmark	Christensen [50]
	50	12				Belgium	Wauters et al. [43]
	400	3			1	The Netherlands	de Boer and Nouws [44]
	45	8				Norway	Nesbakken et al. [51]
	67	1	8[c]	3		China	Tsai and Chen [52]
	48	1			1	Germany	Karib and Seeger [45]
	40	2	4		1	Ireland	Logue et al. [53]
	1278	64	14			Japan	Fukushima et al. [54]
	255	4				Finland	Fredriksson-Ahomaa et al. [55]
	300	6				Norway	Johannessen et al. [30]
	120	14				Germany	Fredriksson-Ahomaa et al. [36]
	60				20	Norway	Grahek-Ogden et al. [32]

[a]Offal, excluding tongue, [b]other pork products, excluding offal, [c]isolates belonging to serotype O:5 and showing autoagglutination activity and calcium-dependent growth.

other studies, pathogenic *yst*-positive *Y. enterocolitica* strains have been isolated from ground beef [29] but not detected in chicken food samples [110].

2.2.2. Contaminated Milk and Milk Products Associated with Human Disease. *Y. enterocolitica* has been isolated from raw milk in many countries, like Australia, Canada, Czechoslovakia, and USA. There were also a few reports on the isolation of this pathogenic strain associated with human disease from pasteurized milk [4, 111]. It may be due to the malfunction in the pasteurization process leading to inadequate treatment or postprocess contamination, or it may be due to the contamination with heat-resistant strains of *Y. enterocolitica*. So, the presence of this pathogen in pasteurized milk should be a cause for concern. However, heat-resistant strains of *Y. enterocolitica* have not been still reported in milk samples.

2.2.3. Other Contaminated Foods Involved in Outbreaks. Strains of *Y. enterocolitica* have been isolated from oysters, mussels, shrimp, blue crab, fish, salad, stewed mushrooms, cabbage, celery, and carrots [112]. In Korea, Lee et al. [113] isolated *ail*-positive *Y. enterocolitica* strain of bioserotype

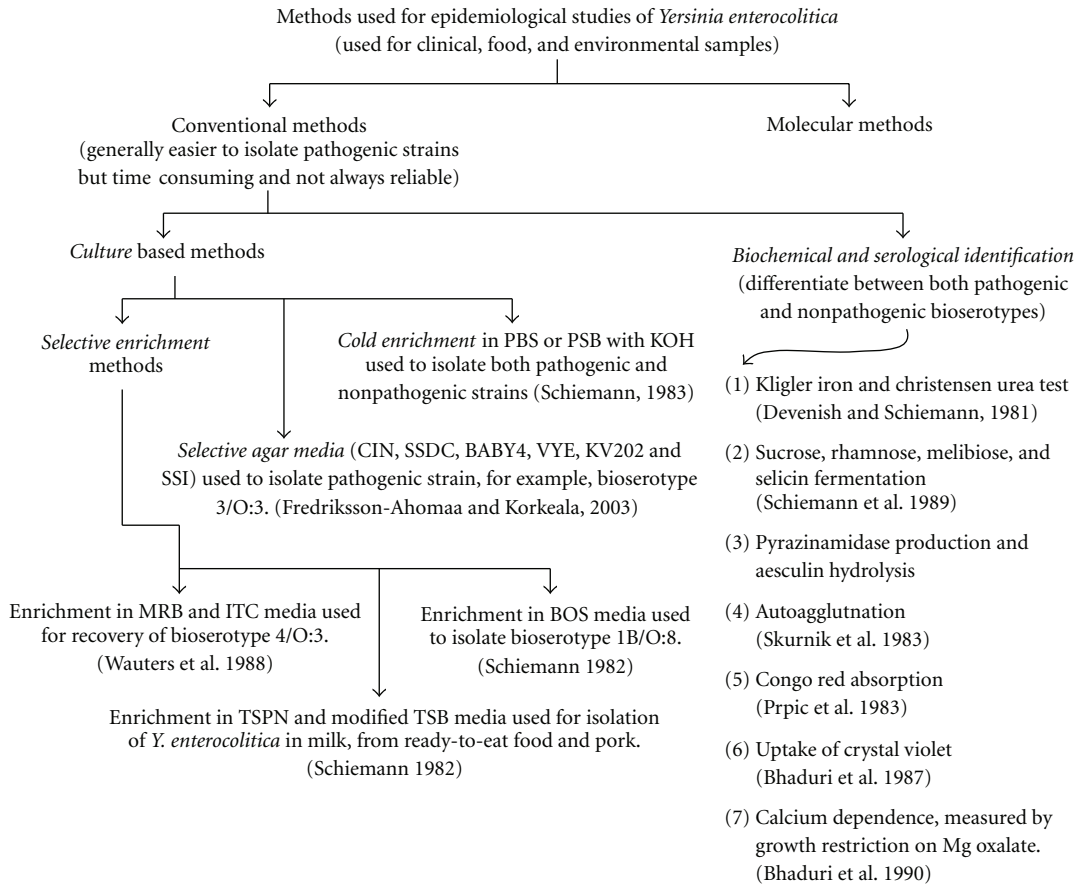

Figure 2: Methods used for epidemiological studies of *Y. enterocolitica*-1. Selective enrichment methods [43]; selective agar media [11]; cold enrichment method [57]; biochemical & serological identification methods [58–63]. (PBS: Phosphate buffered saline; PSB: Phosphate-buffered saline with sorbitol and bile salts; MRB: Modified Rappaport broth containing magnesium chloride, malachite green, and carbenicillin; ITC: Modified Rappaport base supplemented with irgasan, ticarcillin, and potassium chlorate; BOS: Bile-oxalate-sorbose medium; TSB: Tryptic soy broth; TSPN: TSB with polymyxin and novobiocin; CIN: Cefsulodin-irgasan-novobiocin; SSDC: *Salmonella-Shigella* deoxycolate calcium chloride; VYE: Virulent *Yersinia enterocolitica*; SSI: Statens Serum Institute, Copenhagen, Denmark, enteric medium).

3/O:3 from ready-to-eat vegetables, which indicate that vegetables can be a source of human infection. Furthermore, Sakai et al. [34] reported an outbreak of food poisoning by *Y. enterocolitica* serotype O:8 in Japan where salad was proposed the cause of infection. Recently, *Y. enterocolitica* 2/O:9 has been isolated from chicken eggshell surfaces in Argentina [114]. Contamination of the egg surface might have occurred from contact with other *Y. enterocolitica*-contaminated animal products, such as pork product, during collection on farms or during transportation or handling in retail shops.

2.3. Contaminated Environment Reported as Source of Infection. Most of the *Y. enterocolitica* isolates recovered from environmental samples, including the slaughterhouse, fodder, soil, and water, have been nonpathogenic [89, 115–119]. Occasionally, strains of bioserotype 4/O:3 have been isolated from the slaughterhouse [120, 121] and sewage water [50]. Within the environmental sampling sites, drinking water has been relatively widely investigated and revealed to be a significant reservoir for nonpathogenic *Y. enterocolitica*. However, Sandery et al. [35] detected pathogenic *Y. enterocolitica* in environmental water by molecular studies. In a case-control study, untreated drinking water has been reported to be a risk factor for sporadic *Y. enterocolitica* infections in Norway [107]. Recently, Falcão et al. [122] tested 67 *Y. enterocolitica* strains isolated in Brazil from untreated water for the presence of virulence genes. They found that all 38 strains of serotype O:5,27 possessed *inv, ail*, and *ys*t genes, suggesting that untreated water may be responsible for the human infection with *Y. enterocolitica*. In another study, *Y. enterocolitica* O:8 strains have been isolated from stream water in Japan, which indicate that stream water may be a possible infection source for human *Y. enterocolitica* O:8 infections [84, 123].

3. Conclusion

Epidemiological studies of human infection with *Y. enterocolitica* (Table 3) constitute an important element in

Table 3: Epidemiological studies of human infection with *Y. enterocolitica*.

Year	Country	Outcome of the study	References
1981–1990	Georgia	Report of 84 clinical isolates of *Y. enterocolitica*, the most frequently reported serotypes were O:5; O:10,46; O:6,30	Sulakvelidze et al. [89]
1982–1991	The Netherlands	Analysis of clinical information from 261 Dutch patients with gastrointestinal infections caused by *Y. enterocolitica* serotypes O:3 and O:9	Stolk-Engelaar and Hoogkamp-Korstanje [90]
1982[a]	Canada	Outbreak of gastroenteritis among hospitalized patients associated with *Y. enterocolitica* serotype O:5	Ratnam et al. [91]
1982–1985	Canada	Examination of 125 isolates of *Y. enterocolitica*, serotypes O:7,8; O:5; O:6,30, were frequently obtained from symptomatic patients	Noble et al. [92]
1983	Finland	Report of 46 fecal isolates of *Y. enterocolitica*, including two serotypes O:7; O:6, associated with occurrence	Skurnik et al. [60]
1984[a]	Bangladesh	Case report of a fatal diarrheal illness associated with serotypes O:7; O:8	Butler et al. [7]
1984[a]	Hong Kong	Report of *Y. enterocolitica*-associated septicemia in four patients regarding serotypes O:17	Seto and Lau [93]
1984-1985	UK	Report of two nosocomial outbreaks of *Y. enterocolitica* serotypes O:10; O:6 infections in hospitalized children	Greenwood and Hooper [94]
1986[a]	UK	Case report of nosocomial transmission of serotypes O:6,30 associated with gastroenteritis	McIntyre and Nnochiri [95]
1986–1992	Canada	Report of 79 symptomatic children with culture-proven infection, including serotypes O:5; O:6,30; O:7,8	Cimolai et al. [96]
1987	UK	Report of 77 *Y. enterocolitica* strains from patients, including serotypes O:6,30; O:7	Greenwood and Hooper [97]
1987-1988	Australia	Report of 11 cases of *Y. enterocolitica* enteritis, including most frequently serotypes O:6,30	Butt et al. [98]
1987–1989	Chile	A prospective case-control study of infants with diarrhoea in Chile, showing a significantly reported serotypes O:6; O:7,8; O:7; O:10	Morris et al. [99]
1988–1991	Nigeria	Of nine strains of *Y. enterocolitica* obtained from stool samples of children with diarrhoea	Onyemelukwe [100]
1988–1993	New Zealand	Of 918 isolates of *Y. enterocolitica* from symptomatic patients	Fenwick and McCarthy [101]
1968–2000	Brazil	Of 106 strains (selected from the collection of the Yersinia Reference Laboratory in Brazil), 71 were bioserotype 4/O:3, isolated from human and animal clinical material, and 35 were of biotype 1A or 2, isolated from food	Falcão et al. [102]
2002	Iran	Report of 8 cases of *Y. enterocolitica* infection out of 300 children with acute diarrhoea aged 0–12 years who were attending a pediatric hospital in Tehran	Soltan-Dallal and Moezardalan [9]
2002–2004	Nigeria	Detection of *Y. enterocolitica* belonging to bioserotype 2/O:9 in investigating 500 human samples	Okwori et al. [10]
2004	Japan	Report of 16 cases food poisoning due to *Y. enterocolitica* serotype O:8	Sakai et al. [34]
2005–2006	Norway	Investigation of an outbreak involving 11 persons infected with *Yersinia enterocolitica* O:9	Grahek-Ogden et al. [32]
2001-2008	Germany	Almost 90% of *Y. enterocolitica* strains were diagnosed as serotype O:3	Rosner et al. [103]
2009[a]	Iraq	Identification of three children with diarrhoea caused by *Y. enterocolitica* infection	Kanan and Abdulla [8]
2009	Australia	Report of 1 outbreak with 3 cases due to consumption of roast pork contaminated with *Y. enterocolitica*	OzFoodNet sites [104]

[a] Year of publication.

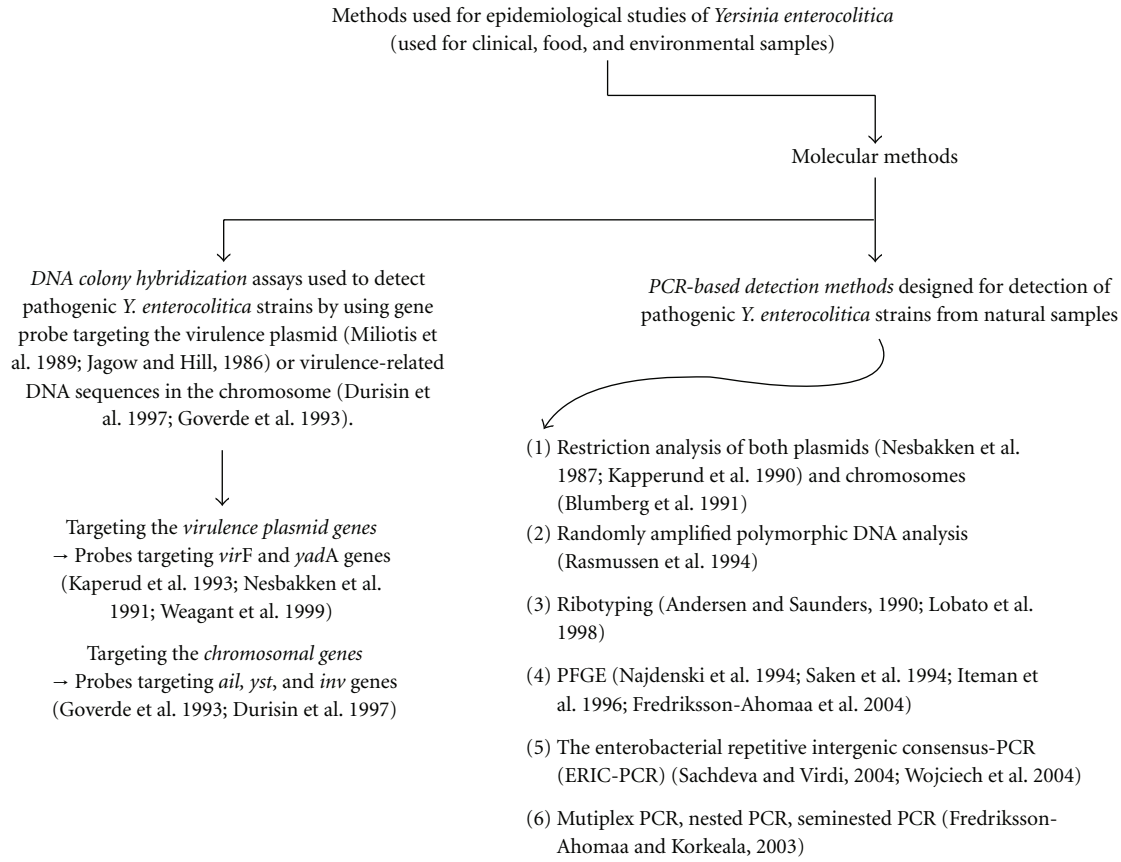

Figure 3: Methods used for epidemiological studies of *Y. enterocolitica*-2. DNA colony hybridization assays [51, 65–70]; PCR based detection methods [11, 71–81]. (*inv*: gene for invasin, an outer membrane protein that is required for efficient translocation of bacteria across the intestinal epithelium; *ail*: gene for adhesin, an outer membrane protein that may contribute to adhesion, invasion and resistance to complement-mediated lysis; *yst*: gene for heat-stable enterotoxin that may contribute to the pathogenesis of diarrhea associated with acute yersiniosis; *virF*: gene for transcriptional activator; *yadA*, gene for *Yersinia* adhesin A; PFGE: pulsed field gel electrophoresis).

the exploitation of apparent sources and contamination routes of human yersiniosis and in the development and implementation of effective control strategies to prevent future outbreaks. Efficient laboratory methods used for epidemiological study are also a vital requirement in *Y. enterocolitica*'s monitoring and control purposes. Molecular methods should be needed with conventional culture methods to provide a better estimation of epidemiology of *Y. enterocolitica* particularly pathogenic strains in natural samples

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Review Article

Yersinia enterocolitica and *Yersinia pseudotuberculosis* Detection in Foods

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Yersinia enterocolitica and *Y. pseudotuberculosis* which can cause yersiniosis in humans and animals are thought to be significant food-borne pathogens and be important as hygiene indicator in food safety. The pathogenic *Y. enterocolitica* serotypes/biotypes are O:3/4 and 3 variant VP negative, O:5, 27/2, O:8/1b, and O:9/2, have been reported worldwide. *Y. pseudotuberculosis* is distributed less widely than *Y. enterocolitica*. Isolation methods usually involve selective and recovery enrichment of the food sample followed by plating onto selective media, confirmation of typical colonies and testing for virulence properties of isolated strains. Recently, DNA-based methods, such as PCR assays, have been developed to detect pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in foods more rapidly, and sensitivity than can be achieved by conventional culture methods. This paper reviews commercially available conventional and PCR-based procedures for the detection of pathogenic *Yersinia* in food. These methods are effective as the isolation and detection methods to target pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in foods.

1. Overview

Food-borne pathogenic *Yersinia* (*Y. enterocolitica* and *Y. pseudotuberculosis*) is facultative anaerobic, gram-negative *Enterobacteriaceae* and is isolated frequently from soil, water, animals, and foods [1–4]. *Y. enterocolitica* causes human infections whose symptoms include diarrhea, terminal ileitis, mesenteric lymphadenitis, arthritis, and septicemia. *Y. pseudotuberculosis* causes mesenteric lymphadenitis, diarrhea, and septicemia in humans. As a psychrophilic organism, *Yersinia* is able to grow at 4°C, and cold chain food products could offer a potential food safety hazard [3, 5, 6]. The pathogenic *Y. enterocolitica* serotypes/biotypes are O:3/4 and 3 variant VP negative, O:5, 27/2, O:8/1b, and O:9/2 have been reported worldwide [7, 8]. In Japan, O:3/3 variant VP negative is the most frequent cause of human yersiniosis [8]. In the United States, despite declining incidences of serotype O:8/1b infections, O:3/4 and O:5, 27/2 infections are on the increase [7]. In Europe, Serotype O:3 and O:9 infections account for over 90% of *Y. enterocolitica* infections. *Y. pseudotuberculosis* is distributed less widely than *Y. enterocolitica* and, although frequently isolated from animals, is rarely isolated from soil, water, and food [9–12].

A large outbreak of *Y. pseudotuberculosis* infection has been reported in Japan [13, 14]. In the Far East including Japan, *Y. pseudotuberculosis* various serotypes (1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, and all that) are isolated from patients with exanthematous systemic infection such as fever, and almost strains isolated produce a superantigenic toxin-designed YPMa encoded by *ypmA* gene [14–16]. In Europe, serotypes (1a, 1b, and 3) have been isolated from patients with gastroenteric symptoms and have an extremely low frequency of isolation [16].

It is therefore important to isolate and identify and differentiate food-borne pathogenic *Yersinia* from nonpathogenic *Yersinia* strains. Isolation methods usually involve enrichment of the food sample followed by plating onto selective media, confirmation of typical colonies, and testing for virulence properties of isolated strains [17]. This method is an effective method which may be employed to *Yersinia*

enterocolitica and *Y. pseudotuberculosis* in foods. The procedure has been used especially to detect the pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in Japan.

2. Procedures Currently to Quantify and Confirm *Yersinia* sp. in Food

The presence of *Y. enterocolitica* and *Y. pseudotuberculosis* in food can be determined quantitatively by a direct culture on selective agar plates. However, confirmatory tests require a combination of cold enrichment, selective enrichment, and subculture on selective agar plates. A conventional protocol for detection and identification of *Y. enterocolitica* and *Y. pseudotuberculosis* from foods is shown in Figure 1. Suspect food samples must however be pretreated to enable successful analysis.

2.1. Pretreatment of Foods. Pretreatment starts with the homogenizing the food sample (25 g) in a stomacher for 2 min with 225 mL of phosphate-buffered saline (PBS) or other cold enrichment medium (see below). The resulting homogenate is used for the direct culture, enrichment culture experiments. For rapid separation and concentration, a 25 g food sample is mixed with 225 mL of 0.02% Tween 20-buffered peptone water (BPW) in a plastic bag (Stomafilter P type; Gunze, Tokyo, Japan), containing a Teflon cloth (40 mesh) on the inside and homogenized in a stomacher for 2 min [18].

2.2. Enumeration of Yersinia sp. by Direct Culture Method. For this procedure, an aliquot of homogenate is inoculated onto selective agar plates (see below) after treatment with an alkali [9, 19]. Alkaline treatment can be achieved by mixing 0.5 mL of homogenate with 0.5 mL of 0.72% KOH in 0.54% NaCl for 30 sec. *Yersinia* is able to resist weak alkaline treatment, and this property is used to select the organism while suppressing background flora such as *Pseudomonas, Proteus* and *Serratia* [20]. It is reported that *Y. enterocolitica* serotypes O:3, O:5, 27, O:8, and O:9 and *Y. pseudotuberculosis* serotype 5a strains in the artificially contaminated pork samples showed comparatively high resistance to KOH, and all *Yersinia* strains were recovered from the pork samples contaminated with more than 10^2 cells per g after direct KOH treatment, without enrichment [9]. However, food samples with low contamination (less than 10^2 cells per g) require an enrichment procedure for successful recovery of *Yersinia*.

2.3. Cold and Selective Enrichment for Recovering Yersinia sp. from Food Samples with Low Contamination Levels. For cold enrichment, an inoculated medium (examples shown below) is incubated at 4°C for three weeks. After 1, 2, and 3 weeks, 0.5 mL of the medium is treated with KOH and inoculated onto selective agar plates. This procedure is useful for enrichment of *Y. enterocolitica* and *Y. pseudotuberculosis*. Being psychrophilic, *Yersinia* can grow at 4°C. However if the medium has low selectivity, environmental *Yersinia* species and other bacteria may also multiply during enrichment [17]. Alkali treatment of the medium helps reduce such non*Yersinia* background flora. Cold enrichment media used for detection of *Yersinia* in food and water samples are

(1) phosphate-buffered saline (PBS; 880 mL of 0.061 M Na_2HPO_4, 120 mL of 0.061 M KH_2PO_4, and 0.85% NaCl) [21],

(2) PBS with 1% mannitol and 0.15% bile salts (PMB) [22],

(3) PBS with 0.5% peptone and 1% sorbitol, 0.15% bile salts (PSB) [23],

(4) PBS with 0.25% peptone and 0.25% mannitol (PMP) [5],

(5) buffered peptone water (BPW, Merck, Germany).

An alternative to cold enrichment is selective enrichment. Selective enrichment uses media containing antimicrobial agents. Several selective enrichment media for isolation of *Y. enterocolitica* at higher temperatures have been developed [17]. Generally, cold enrichment yields higher recovery rates of pathogenic *Y. enterocolitica* than selective enrichment. Moreover, an effective selective enrichment system for *Y. pseudotuberculosis* has not been developed, so its current selective enrichment procedures are especially low.

Nevertheless, in case of outbreaks, selective enrichment procedures for isolation of pathogenic *Y. enterocolitica* are useful for rapid detection and confirmation of the pathogen. In such cases, 9 mL of Irgasan-ticarcillin-potassium chlorate (ITC, Merck, Darmstadt, Germany) is inoculated with 1 mL of medium from cold enrichment and aerobically incubated at 25–30°C for 48 hr [22].

2.4. Rapid Separation and Concentration of Yersinia from Food Samples for Cell Counting and PCR [18]. A conventional protocol for rapid separation and concentration of food-borne pathogens in food samples using filtration, centrifugation, and buoyant density centrifugation (BDC) prior to quantification by viable-cell counting and real-time PCR is shown in Figure 2. A 25 g food sample is mixed with 225 mL of 0.02% Tween 20-BPW in a small plastic bag (Stomafilter P type) and homogenized in a stomacher for 2 min. Approximately 220 mL portions of filtered solutions of the homogenates are placed in sterilized 350 mL glass tubes and centrifuged at 1,880 ×g for 5 min at room temperature, using a swing rotor. The upper portion is transferred to a sterilized 500 mL plastic tube and then centrifuged at 16,000 ×g for 5 min at room temperature. The pellet is then suspended in 1.5 mL of 0.15 M NaCl and centrifuged at 14,500 ×g with a bench-top centrifuge for 5 min at room temperature. The resultant pellet is harvested and used for the second step.

The second step is flotation and sedimentation BDC for purification of food-borne pathogens. In the flotation assay, 0.5 mL portions of sample suspensions are mixed with 1 mL of a 1.050 g/mL Percoll solution (Pharmacia Biotech, Sweden) and centrifuged at 4,500 ×g for 15 min at room temperature. The upper portion, including the food matrix, is carefully removed. For the sedimentation assay, the bottom portion (about 0.5 mL), including organisms, food

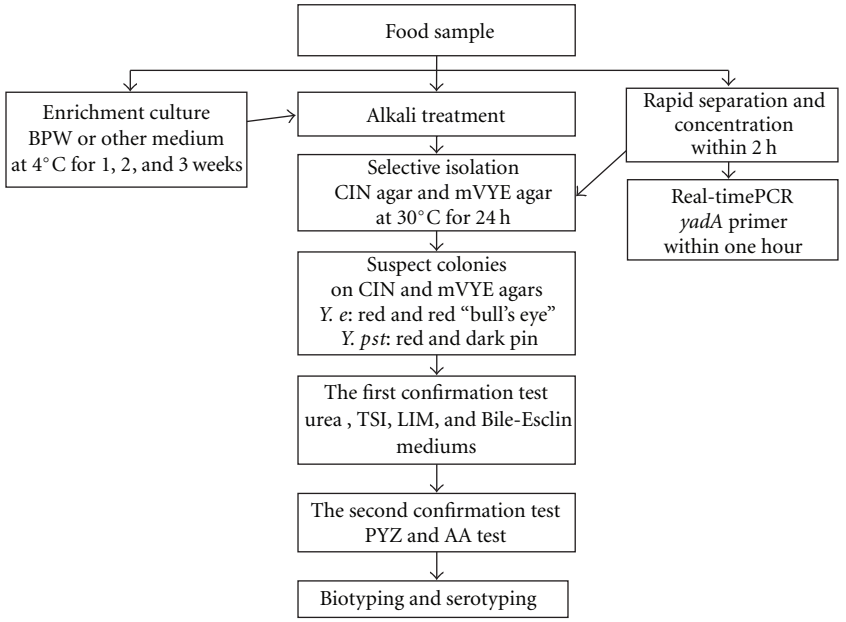

Figure 1: Optimal protocol for detection and identification of *Y. enterocolitica* and *Y. pseudotuberculosis* from foods.

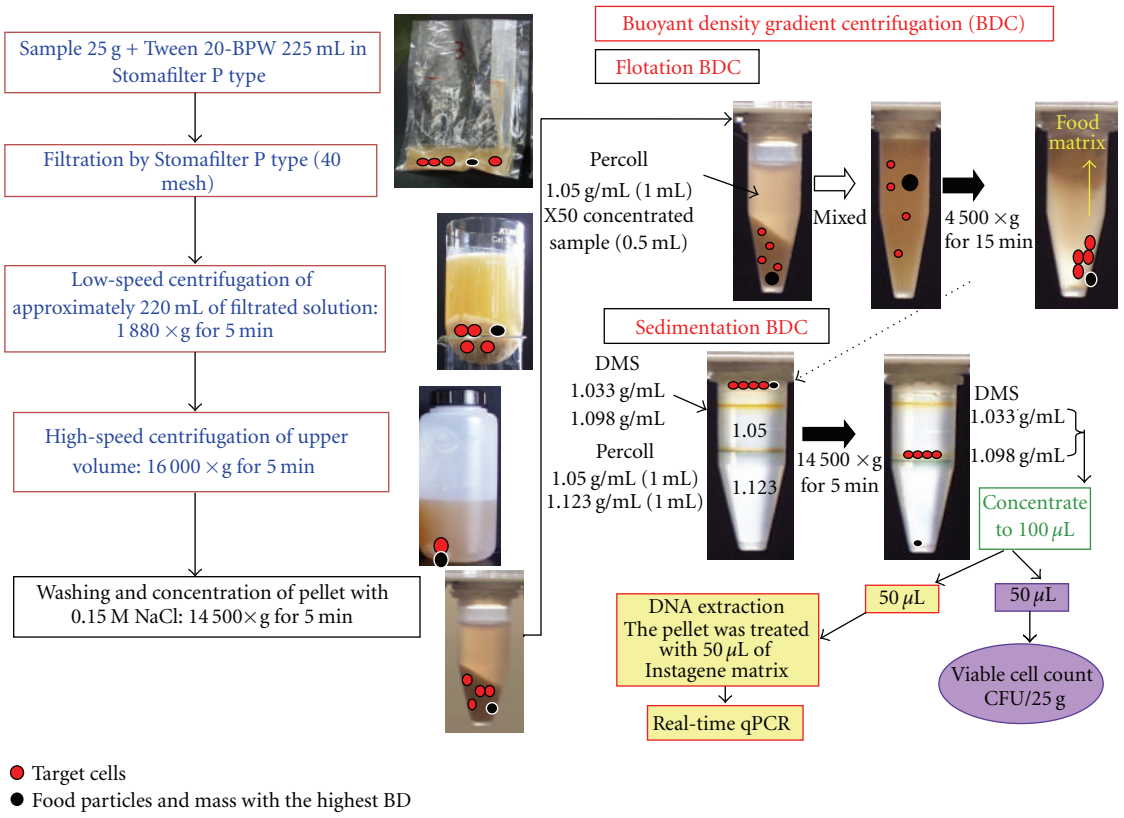

Figure 2: Optimal protocol for rapid separation and concentration of food-borne pathogens in food samples using filtration, centrifugation, and BDC prior to quantification by viable-cell counting and real-time PCR.

Table 1: Confirmation characteristics of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*.

Test Species	Medium	Condition	Characteristic	Reactions									
				Y. enterocolitica						*Y. pseudotuberculosis*			
The first confirmation test	Urea broth	30°C, 24 h	Urea	+						+			
	TSI		Slant/column	yellow/yellow						red/yellow		red/yellow	
			H_2S	−						−		−	
			Gas	−						−		−	
	LIM		Lysin	−						−		−	
			Indole	d						−		−	
			Motility	+,d						+		+	
	Bile-esculin agar		Esculin	+		−				−		+	
The second confirmation test	Pyrazinamidase-test agar	30°C, 48 h	Pyrazinamidase	+		−				−		−	
	TSB	25°C & 37°C, 24 h	Autoagglutination	−		+				+		+	−
Biotype of *Y. enterocolitica* Genetic group of *Y. pseudotuberculosis*				1A	1B	2	3	3 variant VP^-	4	3 VP^-, S	1, 2, 3, 6	5	4
Biotyping test		30°C, 48 h	Lipase	+	+	−	−	−	−	−	−	−	−
			Indole	+	+	(+)	−	−	−	−	−	−	−
			Xylose	+	+	+	+	+	−	+	+	+	+
			Voges-Proskauer (VP)	+	+	+	+	−	+	−	−	−	−
Differentiation test			Sucrose	+	+	+	+	+	+	−	−	−	−
			Rhamnose	−	−	−	−	−	−	−	+	+	+
			Melibiose	−	−	−	−	−	−	−	+	−	−
Serotyping				Others	O:8	O:5, 27 O:9	O:3	O:3	O:3	O:3	O:1 to O:15	O:3	part of O:1, O:5, O:6, O:7, O:9, O:10, O:11, O:12

Pyrazinamidase test is carried out by inoculating a slant of the pyrazinamidase test agar and incubating at 30°C for 48 hours. Then 1 mL of 1% freshly prepared aqueous solution of ammonium ferric sulphate is poured on the slant. After 15 minutes, the reaction is investigated. A brownish pink colour indicates formation of pyrazinic acid and is a positive pyrazinamidase reaction.
Autoagglutination test is carried out by inoculating Trypticase soy broth (TSB; BBL) (or MR-VP medium; Difco) and incubating at 25°C and 37°C for 24 hours. The virulent plasmid-positive strain invariably autoagglutinated in TSB when grown at 37°C but did not at 25°C.

particles, and the mass with the highest buoyant density, is homogenized and then placed on top of two layers (0.6 mL of a 1.050 g/mL Percoll solution and 0.6 mL of a 1.123 g/mL Percoll solution) in a 1.5 mL microtube to which two density markers (orange for 1.033 g/mL and green for 1.098 g/mL) are added. The preparations are centrifuged at 14,500 ×g for 5 min at room temperature, and then using sterile 1-mL pipettes, about 1 mL is taken from the interface between the two density makers and divided into two samples. The sample is added to 1 mL of 0.15 M NaCl in a 1.5 mL microtube.

The preparations are then centrifuged at 14,500 ×g for 5 min. The bottom portions (0.5 mL) are resuspended with 1 mL of 0.15 M NaCl and then centrifuged at 14,500 ×g for 5 min. Each pellet is used for viable-cell counting and DNA extraction with InstaGene matrix (Bio-Rad). One portion of the sample is resolved with 50 μL of 0.15 M NaCl, and then viable-cell counts (CFU/g), which are obtained by culturing each dilution (10 μL) using selective agar plates, are determined for these BDC-lysate pellets (50 μL). The other portion is treated with 50 μL of InstaGene matrix for DNA extraction prior to real-time qPCR by using *yadA* primer for pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*. The total volume of 25 g food sample is reduced to 0.1 mL, and the target organisms in the sample are theoretically concentrated 250-fold within 2 hr.

2.5. Isolation of Yersinia Using Selective Agar Media. Isolation of *Yersinia* and pathogenic *Yersinia* (pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis*) can be done using Cefsulodin-Irgasan-Novobiocin agar (CIN agar, Difco, Oxoid) [20] and CIN agar containing 0.1% esculin and 0.05% ferric citrate (modified virulent *Yersinia enterocolitica* agar (mVYE agar)) [24].

CIN agar is useful to expedite the recovery of *Y. enterocolitica* and mVYE agar to differentiate virulent from avirulent isolates (Figures 3 and 4). The characteristic deep red center ("bull's eye") with a transparent margin and diameter 2–4 mm appearance of *Yersinia* colonies on CIN incubated at 30°C for 24 hr is important for identification and is due to the presence of mannitol. *Yersinia* ferments the mannitol in the medium, producing an acid pH which gives the colonies their red color and the "bull's eye" appearance.

The greatest advantage of mVYE agar is that pathogenic *Y. enterocolitica*, which forms red colonies, is easily differentiated from most nonpathogenic *Yersinia* organisms and other gram-negative bacteria, which form dark-red colonies with a dark peripheral zone as a result of mannitol fermentation and esculin hydrolysis. *Y. pseudotuberculosis*, which forms dark pin colonies as a result of esculin hydrolysis, is easily differentiated from most nonpathogenic *Yersinia* organisms.

The "bull's eye" colonies on CIN agar and red colonies on mVYE agar are suspected to virulent *Y. enterocolitica* (and sometimes *Y. kristensenii*). The red pin colonies on CIN agar and dark-red pin colonies on mVYE agar are suspected to *Y. pseudotuberculosis.*

2.6. The First Confirmation Test for Colonies from Selective Agar Media. Colonies showing typical morphology on CIN and mVYE agars (at least four colonies from each of the agar plates) are selected. The strains are confirmed according to the criteria shown in Table 1. The first confirmation test is carried out by inoculating urea broth, TSI medium, LIM medium, and Bile-esculin agar and incubating at 30°C for 24 hr.

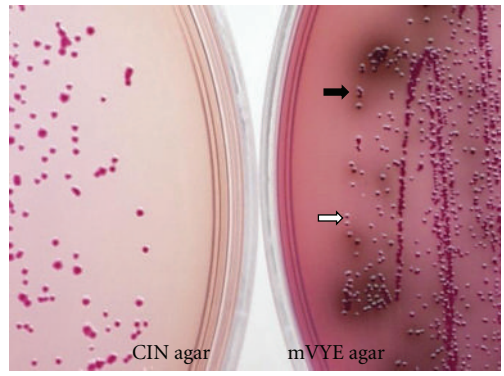

Figure 3: Colonies of *Y. enterocolitica* grown on CIN agar and mVYE agar incubated at 30°C for 24 h. *Yersinia* colonies on CIN form the characteristic deep red center ("bull's eye") with a transparent margin and diameter 2–4 mm. Pathogenic *Y. enterocolitica* serotype O:3/biotype 3 variant VP$^-$ (white arrow) forms red colonies and is easily differentiated from most nonpathogenic *Yersinia* organisms (black arrow) and other Gram-negative bacteria, which form dark-red colonies with a dark peripheral zone as a result of mannitol fermentation and esculin hydrolysis.

Depending on the target organism, colonies are selected for further examination. In the case of *Y. enterocolitica,* colonies that are urea positive; lysine negative in the LIM medium; no gas formation in the TSI medium; glucose positive, sucrose positive; lactose negative (yellow slant and yellow agar column in the TSI medium) should be selected. If the targets are *Y. pseudotuberculosis*, *Y. enterocolitica* biotype 3 VP$^-$, and sucrose negative/serotype O:3, colonies that are glucose positive, sucrose negative, and lactose negative (yellow slant and red agar column in the TSI medium) should be selected for further examination. It should be noted that pathogenic *Y. enterocolitica* strains (serotypes O:3, O:5, 27, O:8, and O:9) are esculin negative while *Y. pseudotuberculosis* strains and nonpathogenic *Y. enterocolitica* strains (biotype 1A/other numerous serotypes) are esculin positive.

2.7. The Second Confirmation Test from the First Confirmation Test. Strains suspected as pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* by the first confirmation tests are selected for the second confirmation tests (pyrazinamidase test [25] and autoagglutination test [26]). For pyrazinamidase test, the strain is inoculated onto pyrazinamidase test agar slants (see below) and incubated at 30°C for 48 hr. For autoagglutination test, the strain is incubated in Trypticase soy broth (TSB; BBL) (or MR-VP medium; Difco) at 25°C and 37°C for 24 hr.

Pyrazinamidase test agar.

Trypticase soy agar (Difco) (30.0 g).

Yeast extract (Difco) (3.0 g).

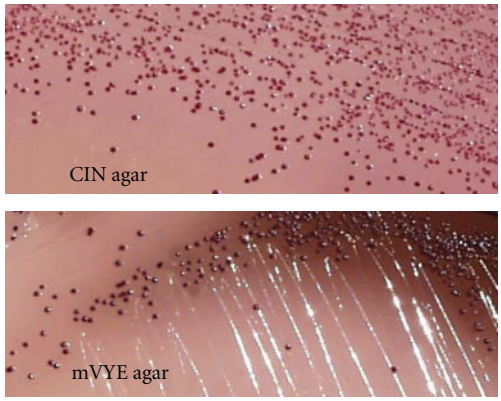

Figure 4: Colonies of *Y. pseudotuberculosis* grown on CIN agar and mVYE agar incubated at 30°C for 24 h. *Y. pseudotuberculosis* forms red pin colonies on CIN agar and dark-red pin colonies on mVYE agar.

Pyrazinecarboxamide (Merck) (1.0 g).

Tris-maleate (0.2 M, pH6) to (1,000 mL).

The 5 mL portions of culture medium are autoclaved and cooled to make slants.

The pyrazinamidase test is to date the chromosomal phenotypical criterion to distinguish potentially pathogenic from nonpathogenic strains. Pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* show negative reactions, and nonpathogenic *Yersinia* strains show positive reactions which turn brownish pink in the presence of ferrous salts (Figure 5). Autoagglutination test is positive on the plasmid for *Yersinia* virulence- (pYV-) positive strains of *Y. enterocolitica* and *Y. pseudotuberculosis* which are incubated at 37°C but not at 25°C (Figure 6). The pYV lost strains which are subcultured, especially at 37°C, and stored, show negative reactions.

2.8. Further Biochemical and Serological Confirmation. Pure strains of suspected pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* are prepared on blood agar or other nutrient agar. The strains are investigated oxidase activity (negative), carried out Gram staining (negative). Then the strains are performed biotyping of *Y. enterocolitica* or genetic grouping of *Y. pseudotuberculosis* according to the criteria shown in Table 1. The serotyping is carried out by slide agglutination using commercial antisera O:3, O:5, O:8, and O:9 for *Y. enterocolitica* (Denka Seiken, Tokyo, Japan), and antisera O:1, O:2, O:3, O:4, O:5, and O:6 (Denka Seiken) for *Y. pseudotuberculosis*. The serotype and subserotype of *Y. pseudotuberculosis* are carried out possibly by O-genotyping using O-antigen gene cluster-specific PCRs [27].

The following parameters can be used to distinguish between *Y. enterocolitica* and other *Yersinia* species: sucrose (positive), rhamnose (negative), melibiose (negative), ornithine decarboxylase (positive) and Voges-Proskauer (VP) positive. However, VP and/or sucrose-negative strains of *Y. enterocolitica* [18, 28, 29] and melibiose-negative strains of *Y. pseudotuberculosis* [16] may occur.

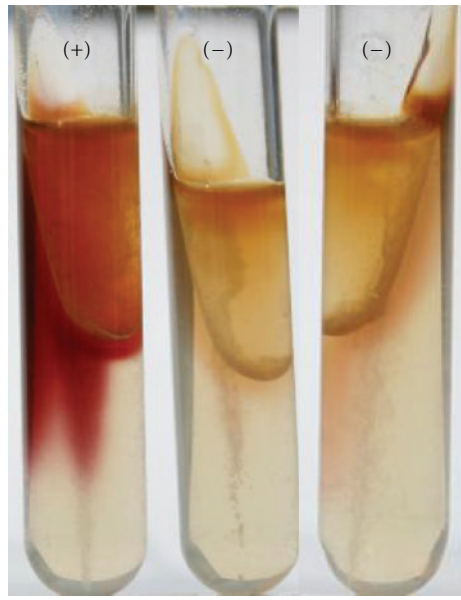

Figure 5: Pyrazinamidase test. A brownish pink color indicates formation of pyrazinic acid and is a positive pyrazinamidase reaction. *Y. enterocolitica* serotype O:5/biotype 1A (a) is positive and *Y. enterocolitica* serotype O:3/biotype 3 variant VP$^-$ (b) and *Y. pseudotuberculosis* serotype 4b (c) are negative reaction.

Y. enterocolitica serotype O:3/biotype 4 is distributed all over the world and is the dominant human pathogenic strain in western countries. However, serotype O:3/biotype 3 variant VP$^-$ is the dominant human pathogenic strain in China, Taiwan, and Japan, and serotype O:3/biotype 3VP$^-$, sucrose negative (S$^-$) is also reported in Japan. Serotype O:5,27, which is reported in the USA, China, and Japan, and Serotype O:9, from the Nordic countries, China, and Japan, belong to biotype 2. Serotype O:8 from the USA and Japan belongs to biotype 1B. Biotype 1A comprises numerous serotypes which have not been associated with human illness and are common in food and the environment.

Y. pseudotuberculosis strains belong to genetic groups 1 to 6 and serotypes 1a, 1b, 1c, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15. The genetic group 3 (Far Eastern systemic-pathogenicity type)/serotypes 1b, 1c, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, 6, 7, 8, 10, and 15 are the human pathogens in Japan, China, and Korea. The genetic group 2 (European gastroenteric-pathogenicity type)/serotypes 1a and 1b and genetic group 5/serotype O:3 are the human pathogens in western countries. Although most strains of *Y. pseudotuberculosis* are melibiose positive, genetic group 4/serotypes O:1, O:5, O:6, O:7, O:9, O:10, O:11, and O:12 (nonpathogenic strains), which are distributed in the environment of Japan, and genetic group 5/serotype O:3 are melibiose negative [16].

2.9. Molecular Detection by PCR for Rapid Detection of Y. Enterocolitica. Using PCR, pathogenic *Y. enterocolitica* can be detected in samples rapidly and with high specificity and

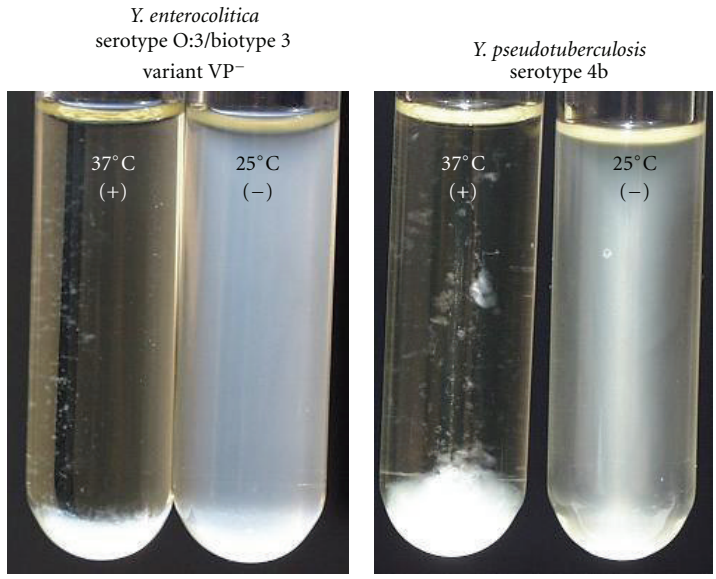

Figure 6: Autoagglutination test. Virulent plasmid-positive strains of *Y. enterocolitica* and *Y. pseudotuberculosis* produced outer membrane protein and autoagglutinate when were incubated in TSB or MR-VP medium at 37°C.

sensitivity [17]. Several PCR assays have been developed to detect pYV-positive *Y. enterocolitica* and *Y. pseudotuberculosis* in clinical, food, and environmental samples. Many of these samples use primers targeting the *yadA* or *virF* gene located on pYV. Because of possible plasmid loss on subculture and storage [30], PCR methods targeting chromosomal virulence genes have also been created for environmental samples. The *ail* gene, located in the chromosome of pathogenic *Y. enterocolitica* strains, and *inv* gene, located in the chromosome of *Y. pseudotuberculosis* strains, are the most frequently used targets. Multiplex PCR method using a mixture of primers against *inv* (5′-TAAGGGTACTATCGCGGCGGA-3′ and 5′-CGTGAAATTAACCGTCACACT-3′), *ail* (5′-ACTCGATGATAACTGGGGAG-3′ and 5′-CCCCCAGTA-ATCCATAAAGG-3′), and *virF* (5′-TCATGGCAGAAC-AGCAGTCAG-3′ and 5′-ACTCATCTTACCATTAAGAAG-3′) [31] has been designed to detect *Y. enterocolitica* and *Y. pseudotuberculosis* in food and water [32].

Real-time PCR is a powerful advancement of the basic PCR technique. At present, the most popular real-time PCR assays are based on "Taqman" and "SYBR Green" approaches. The Taqman system is a 5′-nuclease assay that utilizes specific hybridization of a dual-labelled Taqman probe to the PCR product. The SYBR Green system is based on the binding of the fluorescent SYBR Green dye to the PCR product [33]. Chromosomally encoded *ail* [34] and *yst* [35] genes, the plasmid-borne *yadA* gene [36, 37] and a *Yersinia*-specific region of the 16S rRNA gene [37, 38] have been used in real-time PCR.

Pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* strains yield positive PCR products from the *yadA* gene [39]. Using SYBRGreen real-time PCR assay, the *Tm* values of this *yadA* primer pair (yadA-F1757: 5′-ACGAGTTGACAAAGG-TTTAGCC-3′ and yadA-R1885: 5′-GAACCAACCGCT-AATGCCTGA-3′) are also different between the pathogenic

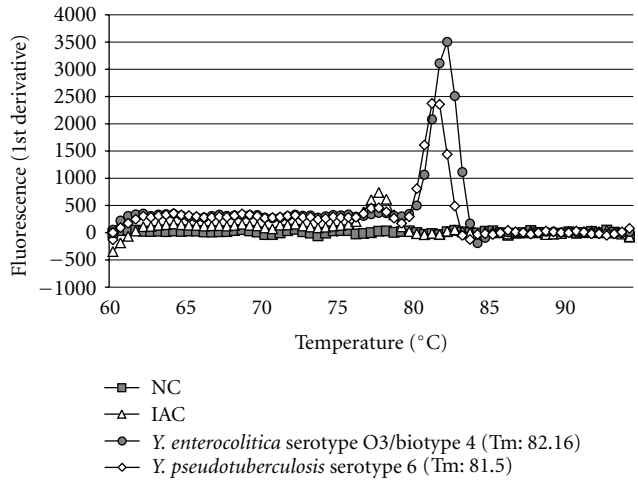

Figure 7: *Y. enterocolitica* and *Y. pseudotuberculosis* dissociation curve in SYBR Green real-time PCR assay.

Y. enterocolitica (82.2°C) and *Y. pseudotuberculosis* (81.5°C) strains (Figure 7). Therefore, this primer pair was confirmed to be useful for detection and differentiation of the two pathogenic *Yersinia* species.

3. Conclusion

Yersinia enterocolitica and *Y. pseudotuberculosis* continue to be important in food safety. While *Yersinia* can survive in many types of food, there is no much information about its the prevalence. This paper covers commercially available conventional and PCR-based procedures for the detection of pathogenic *Yersinia* in food. These methods are effective as the detection methods to target for pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* in foods. However,

development of rapid test methods is needed to facilitate more timely and cost-effective testing.

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Review Article

Virulence Plasmid (pYV)-Associated Expression of Phenotypic Virulent Determinants in Pathogenic *Yersinia* Species: A Convenient Method for Monitoring the Presence of pYV under Culture Conditions and Its Application for Isolation/Detection of *Yersinia pestis* in Food

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In *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, phenotypic expression of virulence plasmid (pYV: 70-kb)-associated genetic determinants may include low-calcium response (Lcr, pinpoint colony, size = 0.36 mm), colony morphology (size = 1.13 mm), crystal violet (CV) binding (dark-violet colony), Congo Red (CR) uptake (red pinpoint colony, size = 0.36 mm), autoagglutination (AA = cells agglutinate), and hydrophobicity (HP = clumping of cells). *Y. pseudotuberculosis* is chromosomally closely related to *Y. pestis;* whereas, *Y. enterocolitica* is chromosomally more distantly related to *Y. pestis* and *Y. pseudotuberculosis*. All three species demonstrate Lcr, CV binding, and CR uptake. The colony morphology/size, AA, and HP characteristics are expressed in both *Y. pseudotuberculosis* and *Y. enterocolitica* but not in *Y. pestis*. Congo red uptake in *Y. pestis* was demonstrated only on calcium-deficient CR magnesium oxalate tryptic soy agar (CR-MOX), whereas this phenotype was expressed on both CR-MOX and low-calcium agarose media in *Y. pseudotuberculosis* and *Y. enterocolitica*. These phenotypes were detectable at 37°C within 24 h in *Y. enterocolitica* and *Y. pseudotuberculosis* but did not appear until 48 h in *Y. pestis* due to its slower growth rate at 37°C. The pYV is unstable (i.e., easily lost under a variety of culture conditions) in all three species but is more unstable in *Y. pestis*. The specific CR uptake by *Y. pestis* in CR-MOX and the delayed time interval to express Lcr and CR uptake provide a means to differentiate *Y. pestis* from *Y. enterocolitica* and *Y. pseudotuberculosis*. These differences in pYV expression in *Y. pestis* can be used for its isolation and detection in food.

1. Introduction

The genus *Yersinia* consists of 11 species, but only *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* are pathogenic to humans. *Yersinia pestis* is considered to be ancestrally related to *Y. pseudotuberculosis*; however, *Y. pseudotuberculosis* behaves phenotypically and clinically like *Y. enterocolitica* [1]. The three species are quite diverse in the diseases they cause; *Y. enterocolitica* and *Y. pseudotuberculosis* induce gastroenteritis when consumed in contaminated food and have been isolated from patients with diarrhea. *Yersinia pestis* is the agent of bubonic plague and can cause oropharyngeal plague as a result of the consumption of inadequately cooked goat and camel meat or handling of meat from infected animals [2–5]. The risk, morbidity, and mortality of contracting plague through the consumption of food deliberately contaminated with *Y. pestis* are currently unknown but potentially real. Furthermore, the identification of multidrug-resistant strains [6] and the potential use of this pathogen for the deliberate contamination of food could cause plague in large populations.

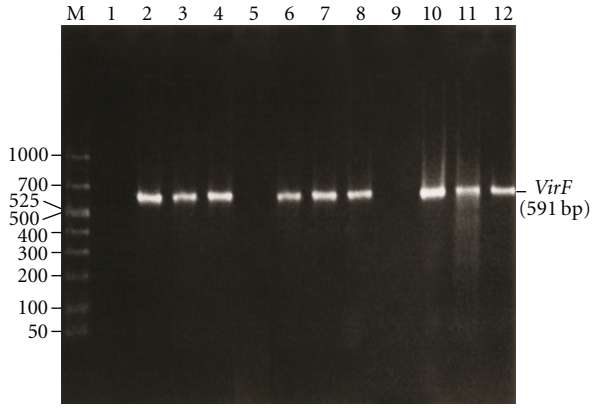

Figure 1: Confirmation of the presence of pYV in the original strains, cells in red pinpoint colonies, and cells in the white border around a red pinpoint colony from CR-MOX by PCR assay targeting a key regulatory gene *virF*, which encodes a transcriptional activator for the expression of pYV-encoded outer membrane protein Yop51. The primer pairs (5′-TCATGGCAGAACAGCAGTCAG-3′ and 5′-ACTCATCTTACCATTAAGAAG-3′) for detection of the *virF* gene (430- to 1020-nucleotide region) amplified a 591 base pair (bp) product from the virulence plasmid. Lane M, 50–1,000 bp ladder marker; lanes, 1, 5, and 9 showing the absence of 591-bp product in cells of the white borders of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis,* respectively; lanes 2, 6, and 10 showing the presence of 591-bp product in the original strains of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* respectively before phenotypic evaluation; lanes 3, 7, and 11 showing the presence of 591-bp product in cells of the red pinpoint colonies of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis,* respectively, and lanes 4, 8, and 12 showing the presence of 591-bp product within cells of red pinpoint colonies surrounded by white border of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* respectively [7].

Three plasmids are involved in the virulence of *Y. pestis*: (a) pYV (virulence plasmid, 70-kb, Yops, type III secretion system), (b) pFra/pMT1 (96.2-kb, murine toxin: phospholipase, F1 capsule-like antigen), and (c) pCP1/pPst/pPla (9.6-kb, plasminogen activator) [8, 9]. Among these plasmids, the pYV-encoded type III secretion system (Yops) promotes cytotoxicity and the common symptoms of plague [8]. The pYV of all three species are of the same size and genetically highly conserved [8, 10–12]. It encodes the ability to target lymph tissues during infection and has genetic determinants essential for infection and overcoming host defense mechanisms [8, 10–12]. In the three species, carriage of pYV is responsible for the calcium-dependent growth phenotype at 37°C. The cultivation of pYV-bearing cells in low-calcium/calcium-deficient media elicits a Mg^{2+}-dependent low-calcium response (Lcr), which results in the production of pYV-encoded virulence-associated antigens (V and W), and a series of released proteins (Yops). The low-calcium response is expressed phenotypically on solid media by the formation of pinpoint colonies [8, 10–12]. Furthermore, pYV in *Y. enterocolitica* has been correlated with several other *in vitro* characteristics, which are phenotypically expressed at 37°C. The well-characterized pYV-associated virulence determinants include colony morphology/size, Lcr, crystal violet (CV) binding, Congo red (CR) uptake, autoagglutination (AA), hydrophobicity (HP), mannose-resistant haemagglutination, expression of surface fibrillae, and serum resistance [11–13]. However, the expression of these physiological traits at 37°C also fosters the loss of pYV and the concomitant disappearance of the associated phenotypes. Since *Y. pestis* and *Y. pseudotuberculosis* have nearly identical chromosomal DNA sequences and are distantly related to pathogenic *Y. enterocolitica* [1, 12, 14], the purpose of this paper is to review whether the phenotypic characteristics induced by pYV are expressed in *Y. pestis* and *Y. pseudotuberculosis* and to determine the growth conditions required for the expression of these phenotypic characteristics. In addition, the detection and isolation of *Y. pestis* by monitoring the presence of pYV-encoded Lcr and CR-uptake virulence phenotypes are discussed.

2. Expression of pYV-Associated Phenotypic Virulence Determinants

In *Y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica,* the expression of phenotypic virulence characteristics is encoded by pYV [7]. A derivative of a clinical *Y. pestis* (KIM5: Kurdistan Iran man) strain lacking the chromosomal 102-kb Pgm locus (pigmentation), but harboring all three virulence plasmids (pYV, pFra/pMT1, and F1) [7, 9], was used for our study. The Pgm locus is only present in *Y. pestis*. This strain is conditionally virulent (a conditional mutant is only infectious if inoculated intravenously) and can be used in a BL2 laboratory facility [7, 9]. This strain shows CR-uptake in *Y. pestis* due to the presence of pYV; whereas, another derivative of a clinical strain of *Y. pestis*, the Kuma strain, contains the chromosomally encoded determinant, Pgm^+ for CR-uptake but lacks pYV [7]. Clinical isolates of *Y. enterocolitica* (serotype O:3; strain GER) and *Y. pseudotuberculosis* (serotype O:1b; strain PB1/+) were also used in our study [7, 13]. The presence of pYV in *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* was confirmed by a PCR assay targeting a key regulatory gene, *virF*, present on pYV (Figure 1, lanes 2, 6, and 10) [15]. The primers (5′-TCATGGCAGAACAGCAGTCAG-3′ and 5′-ACTCATCTTACCATTAAGAAG-3′) for the detection of the *virF* gene (430- to 1020-nucleotide region) amplified a 591-base pair (bp) sequence from the virulence plasmid [15]. *Yersinia pestis* Kuma strain did not show the presence of pYV by the PCR assay.

In our study, the pYV-negative derivatives (P^-) of *Y. pestis* KIM5, *Y. pseudotuberculosis*, and *Y. enterocolitica* were obtained from large flat colonies, which emerged spontaneously from pYV-positive (P^+) cultures growing at 37°C on brain heart infusion agarose with 238 μM Ca^{2+} (BHO) [16] and were used as negative controls. The expression of pYV-encoded genetic determinants in *Y. pestis*, *Y. pseudotuberculosis,* and *Y. enterocolitica* was evaluated [7]. When P^+ and P^- strains were cultivated at 37°C for 24–48 h on a low-calcium brain heart infusion agarose with 238 μM Ca^{2+} (BHO), low-calcium tryptic soy broth agarose with 311 μM Ca^{2+} (TSO), and calcium-deficient magnesium

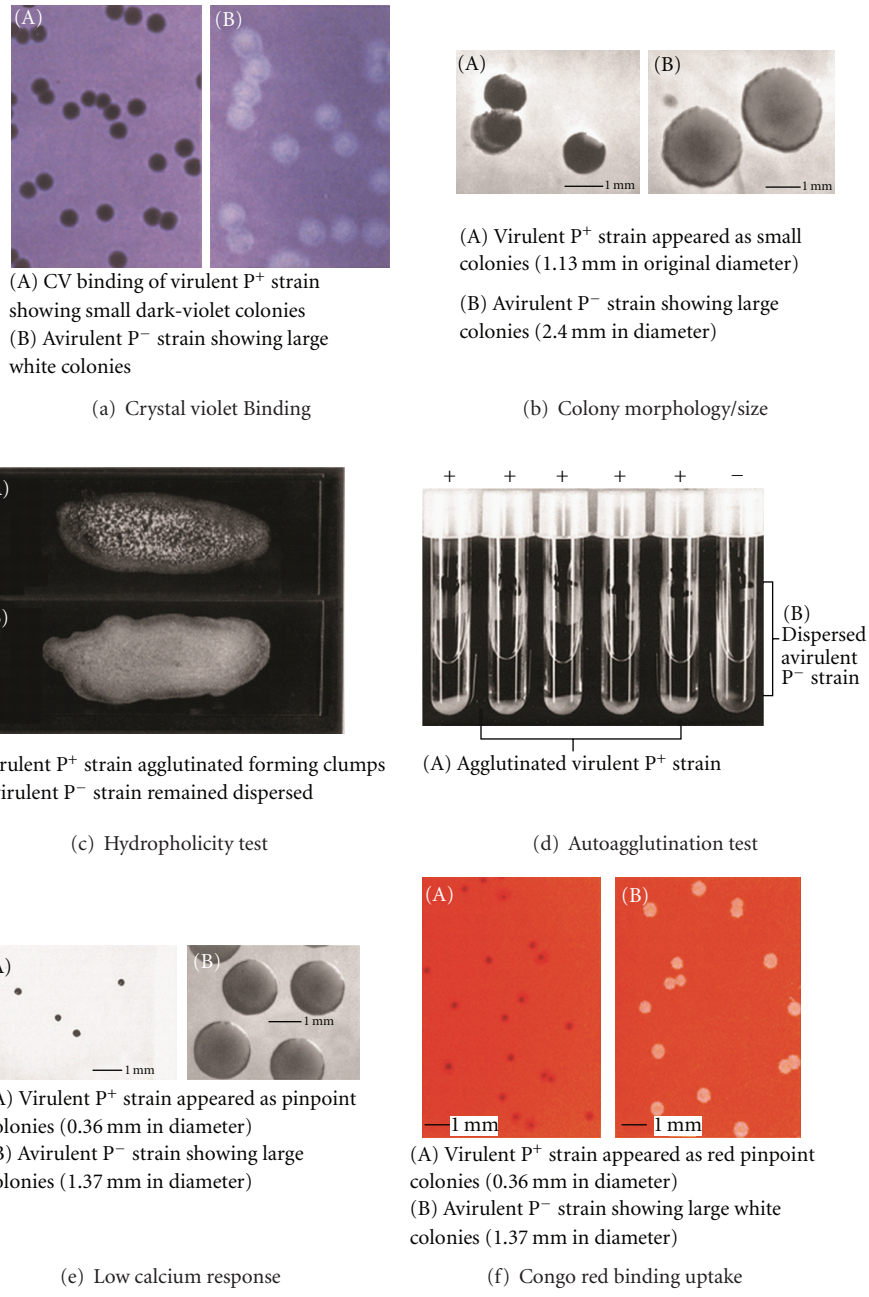

Figure 2: Evaluation of pYV-associated virulent phenotypes of pathogenic *Yersinia* species [7].

oxalate agar with tryptic soy agar (TSA) with 20% D-galactose, 0.25 M sodium oxalate, and 0.25 M magnesium chloride (MOX), the P^+ cells of *Y. enterocolitica* and *Y. pseudotuberculosis* produced pinpoint colonies (0.36 mm in diameter; Figure 2(e)(A) at 24 h, whereas *Y. pestis* P^+ formed pinpoint colonies at 48 h. The P^- cells from each representative strain formed much larger colonies (1.37 mm in diameter; Figure 2(e)(B). The size and colony morphology of each P^+ strain when grown on 75 μg/mL Congo red (CR) containing BHO (CR-BHO), TSO (CR-TSO), and 1% CR containing (CR-MOX) showed identical expression of Lcr as well as CR-uptake (0.36 mm diameter; Figure 2(f)(A) under all these conditions (Table 1). CR-uptake was demonstrated as bright red pinpoint colonies in *Y. enterocolitica* and *Y. pseudotuberculosis* on all three media (Table 2). However, CR-uptake of *Y. pestis* gave a less intense red color as compared to that of *Y. enterocolitica* and *Y. pseudotuberculosis* on CR-BHO and CR-TSO (Table 2). An increase of CR concentration in BHO and TSO to 100 μg/mL, 150 μg/mL, and 200 μg/mL did not increase the color intensity of *Y. pestis* P^+ colonies as compared to colonies of *Y. enterocolitica* and *Y. pseudotuberculosis*. On the basis of color contrast between the bacterial colony and the medium, CR-MOX was more suitable to show CR-uptake in *Y. pestis* as compared to CR-BHO (Table 2). The P^- cells from each representative strain failed to bind CR and formed much larger colonies

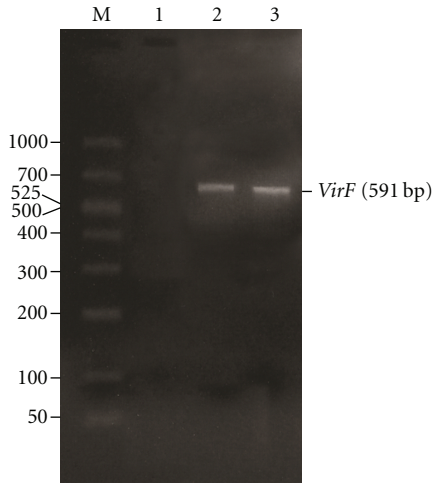

Figure 3: Detection of pYV in cells recovered from red pinpoint colony and subcultured in brain heart infusion broth at 28°C by PCR assay targeting *virF* gene of pYV. The primer pairs (5′-TCATGGCAGAACAGCAGTCAG-3′ and 5′-ACTCATCTTACCATTAAGAAG-3′) for detection of the *virF* gene (430- to 1020-nucleotide region) amplified a 591 base-pair (bp) product from the virulence plasmid. Lane M, 50–1,000 bp ladder marker; lane 1 showing the absence of 591-bp product in *Y. pestis*; lanes 2 and 3 showing the presence of 591-bp product in *Y. enterocolitica* and *Y. pseudotuberculosis,* respectively.

(1.37 mm in diameter; Figure 2(f)(B). The difference of CR-uptake and the difference in timing of the expression of Lcr and CR-uptake in *Y. pestis* facilitate differentiating this species from *Y. pseudotuberculosis* and *Y. enterocolitica*. On calcium-adequate (1500 μM) CR-BHA (brain heart infusion agar) and CR-TSA (tryptic soy agar), colonies of both P^+ and P^- strains of *Y. pseudotuberculosis* and *Y. pestis* remained white or light orange similar to that reported for *Y. enterocolitica* (14, 17). The calcium concentration in CR-BHO (238 μM Ca^{2+}) and CR-TSO (311 μM Ca^{2+}) is relatively low, whereas, in CR-MOX, sodium oxalate is used to sequester calcium leading to a calcium-deficient medium. Thus, the CR-uptake in *Y. pestis* is more dependent on calcium depletion than that of *Y. enterocolitica* and *Y. pseudotuberculosis*. Moreover, *Y. pestis* Kuma, (Pgm^+, pYV^-) failed to bind CR on CR-MOX and formed large white or light orange colonies (1.37 mm in diameter) [7].

That the expression of CR-uptake on CR-MOX is specifically encoded by pYV was further confirmed using a number of derivatives of clinical strains of *Y. pestis* (CDC A1122, CO99.3015, Yokohama, P12, D1, D3, D5, D7, D9, D13, D17) containing Pgm but lacking the pYV [7, 10]. These Pgm^+/pYV^- strains did not bind CR on CR-MOX. These observations indicate that the CR-uptake in *Y. pestis* grown on CR-MOX is associated with pYV. Thus, pYV-encoded CR-uptake is independent of Pgm^+ and that the Pgm locus is not expressed on CR-MOX at 37°C. The CR phenotype is encoded by pYV only on calcium-depleted medium. Thus, CR-uptake in *Y. pestis* grown on CR-MOX is independent of chromosomally encoded CR binding virulence determinants (Pgm^+) and is associated with the presence of pYV.

Another characteristic feature of the CR-uptake in P^+ strains of *Y. enterocolitica* is the appearance of a white opaque circumference around the red center after 48 h of incubation at 37°C [17]. This characteristic colony type was also observed in *Y. pseudotuberculosis* after 48 h of incubation and in *Y. pestis* after 72 h of incubation. The timing of this colonial characteristic is another parameter that can be used for the identification of P^+ strains of *Y. pseudotuberculosis* and *Y. pestis* [7, 17]. The cells in red pinpoint colonies (Figure 1, lanes 3, 7, and 11) and red centered colonies surrounded by a white border (Figure 1, lanes 4, 8, and 12) contained pYV in *Y. pseudotuberculosis* and *Y. pestis* similar to the cells reported in *Y. enterocolitica* [7, 17]. Cells in the surrounding white border (Figure 1, lanes 1, 5, and 9) do not contain pYV as demonstrated by PCR. When the pYV-bearing cells recovered from red pinpoint colonies were subcultured in BHI broth (brain infusion broth) at 28°C for 18 h, *Y. enterocolitica* and *Y. pseudotuberculosis* showed the presence of pYV by PCR (Figure 3, lanes 2 and 3) and pYV-associated phenotypic characteristics, while *Y. pestis* did not harbor pYV (Figure 3, lane 1) under the same conditions (Table 1). This showed that pYV is more stable in *Y. enterocolitica* and *Y. pseudotuberculosis* than in *Y. pestis*. Thus, CR uptake can also be used to isolate viable P^+ cells in *Y. pseudotuberculosis* and *Y. enterocolitica* [7, 17–19].

The flooding of colonies of P^+ strains on BHA, TSA, CR-BHO, CR-TSO, and CR-MOX grown at 37°C with CV solution at a concentration of 100 μg/mL showed that P^+ cells from all three *Yersinia* species bound CV and produced dark-violet colonies (Table 1; Figure 2(a)(A)). The P^- colonies did not bind CV and remained white (Figure 2(a)(B)). The CV- and CR-binding assays can effectively identify individual pYV-bearing colonies from a mixed culture of P^+ and P^- strains [7, 17, 20]. The CR uptake is unrelated to CV binding; these two phenomena are independent since CV uptake is not related to Lcr.

The colony size of P^+ cells in *Y. enterocolitica* and *Y. pseudotuberculosis* was smaller (1.13 mm in diameter; Figure 2(b)(A) than corresponding P^- cells when grown on BHA and TSA at 37°C (2.4 mm in diameter: Figure 2(a)(B) [16], whereas, P^+ and P^- cells of *Y. pestis* were approximately the same size (1.3–1.4 mm SD ± 0.11 in diameter) at 37°C. This may be due to the fact that the optimum growth temperature of *Y. pestis* is 28°C (7, 9, 11, 14). Hydrophobicity by latex particle agglutination was positive (Figure 2(c)(A)) for pYV-bearing *Y. enterocolitica* and *Y. pseudotuberculosis* but negative for P^- cells (Figure 2(c)(B)). *Y. pestis* showed no HP when pregrown cells were tested from CR-BHO, CR-TSO, CR-MOX, BHA, and TSA (Table 1). Thus, HP of *Y. enterocolitica* and *Y. pseudotuberculosis* was expressed in low calcium, calcium-deficient, and calcium-adequate media, indicating that HP is also a non-Lcr property.

The autoagglutination test in Eagle minimal medium supplemented with 10% fetal bovine serum was positive (Figure 2(d)(A)) for pYV-bearing *Y. enterocolitica* and *Y. pseudotuberculosis* but not for P^- cells (Figure 2(d)(B)). *Yersinia pestis* cultures failed to autoagglutinate (Table 1). In both the HP and AA tests, P^- strains were negative for the three species. The explanation for the absence of expression

Table 1: Comparison of selected phenotypic expression of pYV-bearing *Y. enterocolitica, Y. pseudotuberculosis,* and *Y. pestis* (adapted from [7]).

Organism[a]	Strain	CM[b]	CV binding[c]	Lcr[d]	CR-uptake[e]	AA[f]	HP[g]	Plasmid[h]
Y. enterocolitica	GER	+	+	+	+	+	+	+
Y. enterocolitica-RE	GER	+	+	+	+	+	+	+
Y. enterocolitica-C	GER	−	−	−	−	−	−	−
Y. pseudotuberculosis	PB1/+	+	+	+	+	+	+	+
Y. pseudotuberculosis-RE	PB1/+	+	+	+	+	+	+	+
Y. pseudotuberculosis-C	PB1/+	−	−	−	−	−	−	−
Y. pestis	KIM5	−	+	+	+	−	−	+
Y. pestis-RE	KIM5	−	−	−	−	−	−	−
Y. pestis-C	KIM5	−	−	−	−	−	−	−
pYV-less *Y. pestis*	Kuma	−	−	−	−	−	−	−

[a]Cells recovered from red pinpoint colonies and subcultured in BHI broth at 28°C are designated as RE. The pYV-negative strains of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* are designated as C (cured).
[b]CM: colony morphology. On calcium-adequate BHA (1500 μM Ca^{2+}), and TSA (1400 μM Ca^{2+}) the P^+ cells appeared as small colonies (1.13 mm in diameter) as compared to larger P^- colonies (2.4 mm in diameter).
[c]CV binding: crystal violet binding. The P^+ cells appeared as small dark-violet colonies, and the P^- cells showed large white colonies on calcium-adequate BHA (1500 μM Ca^{2+}) and TSA (1400 μM Ca^{2+}), low-calcium CR-BHO (238 μM Ca^{2+}), CR-TSO (311 μM Ca^{2+}), and calcium-deficient CR-MOX.
[d]Lcr: low calcium response/calcium-dependent growth. P^+ cells appeared as pinpoint colonies (0.36 in diameter), and P^- cells appeared large colonies (1.37 in diameter) on low-calcium CR-BHO (238 μM Ca^{2+}), CR-TSO (311 μM Ca^{2+}), and calcium-deficient CR-MOX.
[e]CR-Uptake: Congo red-uptake. The P^+ cells appeared as red pinpoint colonies (0.36 in diameter), and the P^- cells appeared large white or light orange colonies (1.13 mm in diameter) on calcium-deficient CR-MOX.
[f]AA: autoagglutination. The P^+ cells agglutinated. The P^- cells remained dispersed.
[g]HP: hydrophobicity by latex particles. The P^+ cells formed clumps showing hydrophobicity. The P^- cells remained dispersed.
[h]Plasmid: presence of 70-kb pYV by PCR assay.

Table 2: Effect of media on CR-uptake in pYV-bearing *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* (adapted from [7]).

Organism[a]	Strain	CR-BHO	CR-TSO	CR-MOX
Y. enterocolitica	GER	+	+	+
Y. enterocolitica-RE	GER	+	+	+
Y. enterocolitica-C	GER	−	−	−
Y. pseudotuberculosis	PB1/+	+	+	+
Y. pseudotuberculosis-RE	PB1/+	+	+	+
Y. pseudotuberculosis-C	PB1/+	−	−	−
Y. pestis	KIM5	−	−	+
Y. pestis-RE	KIM5	−	−	−
Y. pestis-C	KIM5	−	−	−
pYV-less *Y. pestis*	Kuma	−	−	−

[a]Cells recovered from red pinpoint colonies and subcultured in BHI broth at 28°C are designated as RE. The pYV-negative strains of *Y. enterocolitica Y. pseudotuberculosis,* and *Y. pestis* are designated as C (cured).
Low-calcium: CR-BHO (238 μM Ca^{2+}) and CR-TSO (311 μM Ca^{2+}). CR-MOX (calcium deficient).

of the HP and AA phenotypic characteristics under the conditions described above in *Y. pestis* may be due to the lack of synthesis of pYV-associated surface factors essential for HP and AA or due to a structural/regulatory variability of pYV [21].

In conclusion, of the six pYV-associated phenotypes evaluated, only three phenotypes (Lcr, CR-uptake, and CV binding) were expressed in *Y. pestis*, while all six properties were expressed in *Y. enterocolitica* and *Y. pseudotuberculosis*. This differential expression of pYV-encoded phenotypes may be attributed to *in vitro* assay conditions although pYV is genetically highly conserved in all these species [6, 12, 14, 21]. Thus, the pYV-encoded phenotypes can be used as virulence markers for these pathogens [7, 10, 11, 13]. Although the chromosomal DNA sequence showed that *Y. pestis* and *Y. pseudotuberculosis* are nearly identical and closely related [1, 14], the latter exhibits the same expression of pYV-associated phenotypes as the more distantly related *Y. enterocolitica* and shows similar characteristics and clinical symptoms [1].

3. Procedure to Monitor the Presence of pYV in *Y. pestis* Cells during Storage and Culturing by Using the Lcr-CR-Uptake Techniques

The well-characterized pYV-associated virulence determinants can be used to determine plasmid maintenance, for isolation/detection, and as an indication of virulence for various serotypes of pYV-bearing *Y. enterocolitica* in food

[11, 17–20, 22–25], as well as to determine the presence of pYV in *Y. pestis* and *Y. pseudotuberculosis* [7]. The pYV is unstable in all three pathogens, and the loss of pYV after cultivation or during food processing results in avirulent clones (not lethal to mice; do not cause plague) [7, 11, 13, 26–30]. Repeated transfer of cultures, extended storage at 4°C or −20°C, and laboratory manipulation, as well as subculturing of *Y. pestis* at temperatures >30°C leads to the loss of pYV [7, 29, 30]. Moreover, pYV is more unstable in *Y. pestis* (Figure 3, lane 1) than in of *Y. enterocolitica* and *Y. pseudotuberculosis* (Figure 3, lanes 2 and 3) [7, 29, 30]. The loss of pYV leads to the eventual overgrowth by cells lacking pYV and results in the loss of virulence and the concomitant disappearance of the pYV-associated virulence characteristics [7, 13, 25, 27, 28].

In a study on the growth of *Y. pestis* in ground beef, it was found that the cultures lost pYV during preparation of the inoculum [29]. It was not possible to maintain pYV in cells from the stock cultures using the standard procedures developed previously [13]. Thus, it was difficult to perform a study with *Y. pestis,* which reflected the actual behavior of pYV-bearing *Y. pestis*. In ground beef, the growth rates of pYV less cells were 0.096 and 0.287 CFU/h at 10 and 25°C, respectively; [30] whereas, for pYV-bearing cells, the growth rates were 0.057 CFU/h and 0.233 CFU/h at 10 and 25°C, respectively [29, 30]. The difference in growth rate between pYV-positive and pYV-negative strains of *Y. pestis* was more pronounced at lower temperatures. There was no growth of the pYV-bearing strain at 0 and 4°C as compared to the growth rates of pYV-negative strains of 0.003 and 0.016 CFU/h at 0 and 4°C, respectively, in ground beef [29]. Therefore, the lack of pYV leads to a faster growth rate and does not represent the true growth rate of the pYV-bearing strain. Hence, it is very important to maintain pYV in *Y. pestis* to properly study the growth behavior of a pYV-bearing strain in order to develop a growth model for this pathogen in food. The unstable nature of pYV in *Y. pestis* necessitates an examination for the presence of pYV and its virulence characteristics throughout laboratory manipulation and investigations.

Bhaduri et al. [30] developed a procedure to monitor the presence of pYV in *Y. pestis* cells during storage and culturing by using the Lcr-CR-binding techniques [7, 30], PCR assays, and the expression of pYV-associated virulence characteristics. It is essential to confirm the presence of pYV in the experimental culture by demonstrating that virulence-associated phenotypes were present and to confirm the presence of the pYV-encoded *virF* gene by a PCR assay (Figure 4, lane 3) [7, 15, 30]. The procedures for monitoring the presence of pYV and differentiating pYV positive clones from pYV-negative colonies during laboratory investigations are outlined in Table 3 [30]. As described in Table 3, the first step is to culture *Y. pestis* on CR-MOX and CR-BHO to isolate pYV-bearing clones from the frozen stock culture. The pYV-positive colonies appeared as red pinpoint colonies (0.36 mm in diameter) showing both Lcr and CR-uptake whereas pYV-negative colonies appeared as much larger white or orange colonies (1.37 mm in diameter) [7, 17, 30]. Colony morphology and CR-uptake were used to differentiate between pYV-positive clones and pYV-negative colonies. The Lcr and CR positive clones were further confirmed as pYV positive by the PCR assay (Figure 4, lane 4: CR-MOX and lane 5: CR-BHO) and by pYV-associated Lcr, CR uptake, and CV-binding phenotypes (see Table 3). These pYV-bearing clones were inoculated into BHI broth for the preparation of frozen and working stock cultures as described in Table 3. Before frozen storage and preparation of working stock cultures, the culture prepared in BHI broth at 28°C was tested for the presence of pYV and its virulence-associated phenotypes (Figure 4, lane 6). The Lcr-CR-positive clones on CR-MOX were used as working stock cultures and could be used for 15 days for laboratory studies. After that period of storage, the red pinpoint colonies of *Y. pestis* lost pYV (Figure 4, lane 11). The CR-BHO medium was also successfully used to ensure the selection of pYV in *Y. pestis* although CR-uptake was not as intense as on CR-MOX. The Lcr-CR positive clones were used as working stock cultures from CR-BHO and could be stored for 30 days at 2°C. To ensure the validity of this procedure for selecting pYV in *Y. pestis* cells, we also examined and monitored pYV stability during the subculturing of pYV-bearing cells in BHI broth, CR-MOX, and CR-BHO. *Yersinia pestis* from stock cultures stored at 2°C on CR-MOX and CR-BHO were subcultured as explained in Figure 5 [30]. The presence of pYV in *Y. pestis* cells in each medium and

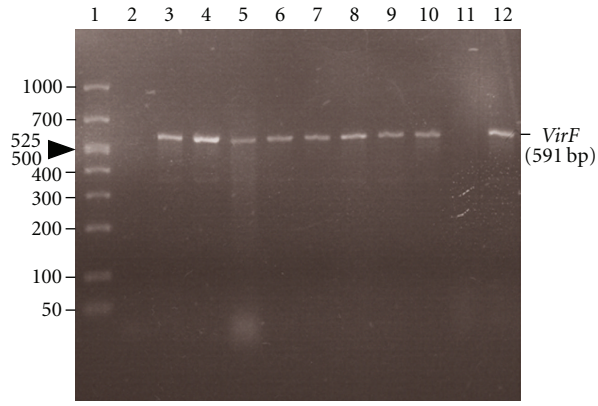

Figure 4: Confirmation of presence of pYV of *Y. pestis* in the original strain before subculturing, and CR-positive clones from CR-MOX, CR-BHO, and BHI broth using PCR assay targeting a key regulatory gene *virF* from pYV. The primer pairs (5′-TCATGGCAGAACAGCAGTCAG-3′ and 5′-ACTCATCTTACCATTAAGAAG-3′) for detection of the *virF* gene (430- to 1020-nucleotide region) amplified a 591-bp-product from the virulence plasmid. The Lcr-CR$^+$ clones showed the presence of 591 bp products from pYV (lanes 2–10 and 12). Lane 1, 50–1,000 bp ladder marker; lane 2, negative control with no template; lane 3, original KIM5 strain as positive control; lanes 4, 5, Lcr-CR$^+$ colonies from the CR-MOX and CR-BHO respectively (Figure 5 ; no. 1); lane 6 BHI broth (Figure 5; 1st passage; no. 2); lane 7 stock culture on CR-MOX (no. 3; 1st passage); lane 8 stock culture on CR-BHO (no. 3; 1st passage); lane 9 BHI broth (no. 4, 2nd passage from CR-MOX); lane BHI broth 10 (no. 4, 2nd passage from CR-BHO); lane 11 (no. 5, 2nd passage on CR-MOX) showing the absence of 591-bp product, and lane 12 (no. 5, 2nd passage on CR-BHO) [30].

Table 3: Isolation and maintenance of pYV in *Y. pestis* [30].

Day 1
(i) Frozen stock cultures were streaked onto CR-MOX and CR-BHO.
(ii) Plates were incubated at 37°C for 48 h for differentiation and isolation of pYV-bearing cells from pYV-less cells.
Day 3
(i) Using a stereomicroscope, red pinpoint colonies were examined to ensure Lcr and CR uptake. Using a sterile loop, 2-3 red pinpoint colonies were then inoculated into sterile 10 mL of BHI broth.
(ii) The broth was inoculated and incubated at 28°C for 18–24 h.
Day 4
(i) The overnight culture was divided into three portions: frozen stock cultures, working stock cultures, and cells used for PCR assay and for expression of pYV-encoded virulent phenotypic characteristics including Lcr, CR uptake, and CV binding.
(ii) Frozen stock cultures: 5 mL of overnight culture was mixed with equal portions of BHI broth and 20% glycerol and dispensed into 500 μL portions for storage at −80°C.
(iii) Working stock cultures: using a 10 μL loop, cells were streaked on CRMOX and CR-BHO. The plates were incubated for 48 h at 37°C. Plates were then stored at 2°C for future use. Plates can be stored for 15 days for CR-MOX and 30 days for CR-BHO.
(iv) PCR assay: 1 mL portion of cells was centrifuged, and DNA was prepared for PCR assay. Presence of pYV was confirmed by PCR assay targeting the *virF* gene in pYV.
(v) The presence of pYV was also confirmed by demonstrating expression of phenotypic virulence characteristics including colonial morphology, CV binding, Lcr, and CR binding.

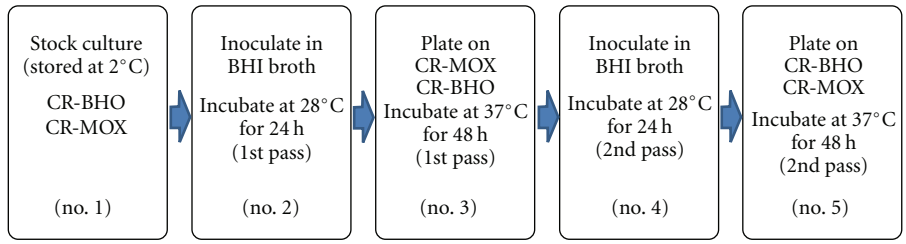

Figure 5: Confirmation of pYV in *Y. pestis*, [30].

after each passage was monitored and confirmed at every step of culture transfer (no. 2–5) by the PCR assay for pYV and by the expression of pYV-associated phenotypic virulence characteristics, including Lcr, CR uptake, and CV binding. The PCR data for the presence of pYV is shown in Figure 1 (lanes 7–10). PCR results confirm that primers amplified a 591-base pair (bp) product from pYV (*virF* gene) for each phase of the culture as described above, and all PCR-positive clones on CR-MOX and CR-BHO showed their virulent phenotypic characteristics including Lcr, CR-uptake, and CV binding. The presence of the *virF* gene demonstrates the presence of pYV, which confers pYV-associated phenotypes.

In conclusion, the described procedure provides a method to ensure the selection of pYV-bearing strains of *Y. pestis* and for studying pYV-bearing *Y. pestis* without losing pYV during experimental procedures [30]. Although CR-BHO is a better medium for subculturing pYV-bearing *Y. pestis*, the pYV-bearing red pinpoint colonies are more easily detectable on CR-MOX due to more intense absorption of CR in the cells [7]. Hence, the use of CR-MOX for the preparation of stock cultures and to monitor the selection of pYV is recommended for investigation on the growth of pYV-bearing *Y. pestis* in food. Thus, this procedure will allow only the Lcr-CR-positive pYV-bearing clones to be used to study growth behavior, growth models, and related studies in food.

4. Application of CR-MOX for Isolation/Detection of *Yersinia pestis* in Food

Yersinia pestis can cause oropharyngeal plague as a result of the consumption or handling of meat from infected animals [2–5]. Thus, food intentionally contaminated by *Y. pestis* could have a significant role in the dissemination of human plague. Existing microbiological media designed for the selective isolation/detection of *Y. pestis* in food based on phenotypic analysis were found to be unsatisfactory. The purpose of this section is to review the development of alternative methods for identification/isolation of pYV-bearing *Y. pestis* based on the ability of *Y. pestis* to bind CR on calcium-depleted CR-MOX under specific conditions.

At present, the World Health Organization (WHO) [31] recommends the use of brain heart infusion (BHA) sheep blood agar and MacConkey agar for the isolation of *Y. pestis*. These growth media are suitable for sterile food;

however, the isolation of *Y. pestis* from nonsterile foods is complicated by the presence of background flora competing for nutrients in the medium. Thus, the numerous colonies grown on these nonselective media require additional testing for the identification of the pathogen. MacConkey agar possesses a certain degree of selectivity; but the presence of CV and bile salt restricts the growth of *Y. pestis* [32]. *Y. pestis* strains exhibit slow or no growth *in vitro* on both cefsulodin-irgasan-novobiocin (CIN) agar and irgasan-nystatin agar [32] selective media when tested in our laboratory [7]. This may be due to the levels of selective substances used in this media. The colonies formed on selective media require further tests to identify them as *Y. pestis*. These tests are time consuming, costly, and labor intensive since a large number of presumptive colonies must be screened.

The calcium concentration in CR-BHO (234 μM Ca^{2+}) is relatively low, whereas in CR-MOX, sodium oxalate is used to sequester the calcium, making the medium calcium deficient [7, 16]. The comparison of CR uptake on calcium deficient CR-BHO and calcium-depleted CR-MOX among *Y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica* showed that this virulent phenotype is seen in pYV-positive strains of *Y. pestis* only when plated on calcium-depleted CR-MOX [7]. Thus, the CR uptake in *Y. pestis* is more dependent on calcium depletion than that of *Y. enterocolitica* and *Y. pseudotuberculosis*. Therefore, specific CR uptake on CR-MOX by *Y. pestis* can be used to differentiate *Y. pestis* from *Y. enterocolitica* and *Y. pseudotuberculosis* [7]. This would provide diagnostic value as follows: the suspected food samples are plated on CR-BHO and CR-MOX. If the colonies show CR uptake only on CR-MOX at 37°C after 48 h of cultivation, then those CR^+ colonies can be isolated and identified as *Y. pestis* strains [7]. This technique will enhance the isolation/detection of *Y. pestis* strains in the presence of competing microflora by the proper selection of media and incubation times. The CR^+ *Y. pestis* clones can be further confirmed by PCR targeting the *Y. pestis* specific plasmid-encoded plasminogen activator gene [33]. To show the specificity of CR uptake by *Y. pestis* on CR-MOX, several species of bacteria including a number of foodborne pathogens were tested. These non-*Yersinia* species did not form red pinpoint colonies and did not form a white border around the red center of the colony on CR-MOX [7, 17]. Furthermore, this method of isolation/detection for *Y. pestis* in food was verified by recovering the organism from artificially contaminated sterilized ground beef [29]. Thus, CR uptake on CR-MOX by *Y. pestis* provides a microbiological method for the isolation/detection of this pathogen. In conclusion, the specific CR uptake of *Y. pestis* in a calcium-deficient medium provides a screening medium to isolate, detect, and differentiate this pathogen from *Y. enterocolitica* and *Y. pseudotuberculosis*, and this method is also applicable to food.

Disclosure

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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Review Article

Pathogenesis of *Y. enterocolitica* and *Y. pseudotuberculosis* in Human Yersiniosis

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Yersiniosis is a food-borne illness that has become more prevalent in recent years due to human transmission via the fecal-oral route and prevalence in farm animals. Yersiniosis is primarily caused by *Yersinia enterocolitica* and less frequently by *Yersinia pseudotuberculosis*. Infection is usually characterized by a self-limiting acute infection beginning in the intestine and spreading to the mesenteric lymph nodes. However, more serious infections and chronic conditions can also occur, particularly in immunocompromised individuals. *Y. enterocolitica* and *Y. pseudotuberculosis* are both heterogeneous organisms that vary considerably in their degrees of pathogenicity, although some generalizations can be ascribed to pathogenic variants. Adhesion molecules and a type III secretion system are critical for the establishment and progression of infection. Additionally, host innate and adaptive immune responses are both required for yersiniae clearance. Despite the ubiquity of enteric *Yersinia* species and their association as important causes of food poisoning world-wide, few national enteric pathogen surveillance programs include the yersiniae as notifiable pathogens. Moreover, no standard exists whereby identification and reporting systems can be effectively compared and global trends developed. This review discusses yersinial virulence factors, mechanisms of infection, and host responses in addition to the current state of surveillance, detection, and prevention of yersiniosis.

1. Introduction

Yersiniosis is typically a self-limiting, gastrointestinal disease of global concern. However, despite the known association of the causative agents (*Y. enterocolitica*, YE, and very rarely *Y. pseudotuberculosis*, YPT) with both gastroenteritis and extraintestinal infections, it remains a poorly understood disease. Sporadic cases are still reported in which food is not suspected as the source of infection, and isolation from contaminated food sources is often problematic. Because yersiniosis is considered relatively uncommon and YE and YPT are ubiquitous, food and water supplies are not regularly monitored for these bacterial pathogens. However, the ability of the yersiniae to persist in a nonculturable but viable state in natural samples [1] and to grow and thrive at refrigeration temperatures (~4°C) suggests that their contribution to disease might be underappreciated.

1.1. YE Infections. The major causative agent of yersiniosis is the gram-negative, zoonotic bacterial pathogen, YE, which is typically transmitted via the fecal-oral route [2]. The closely related YPT can also cause yersiniosis, but human YPT infections are less frequent than those caused by YE. Yersiniosis has been observed on all continents [3] but is most common in European countries. Some of the challenges associated with linking yersiniosis to its source of contamination are attributable to the heterogeneity of yersiniae populations within a plethora of environments and reservoirs including: soil, water, and a variety of animals.

Yersiniosis is an important infection in European brown hares [4] and has additionally been detected in Canadian beavers, snowshoe hares, and muskrats [5]. Additionally, YE and YPT have been isolated form bats in Germany [6]. More relevant to humans is the prevalence of the yersiniae in animal food sources, particularly pigs and pork products [7–9], and more recently in domestic farm dogs in China [10]. Further complicating the picture of disease transmission, a recent study found that wild rodents on a European pig farm tested positive for YE, suggesting that rodents might serve as interspecies carriers between reservoirs [11]. YE has also been isolated from flies found in farm piggeries and kitchens [12], suggesting that arthropod vectors/insects might play a role in the transmission of the enteric yersiniae between animals and humans. Flies might also facilitate the spread of nosocomial infections which is of particular concern because there is at least one report of flies in Libyan hospitals carrying antibiotic-resistant strains of bacteria belonging to the *Enterobacteriaceae* family [13]. The major source of yersiniosis is swine, but recent isolates from contaminated chicken, milk, tofu, and water have also been reported [8, 14].

In healthy, immunocompetent individuals, yersiniosis symptoms range from mild, self-limiting diarrhea to mesenteric lymphadenitis. However, in immunocompromised individuals chronic conditions such as reactive arthritis have also been observed [15]. YE infection is generally established via digestion of contaminated food or water followed by bacterial adherence to small intestinal epithelial cells and eventual crossing of the intestinal barrier *via* M cells [16]. Subsequently, YE bacilli replicate in Peyer's patches and can sometimes spread to more distant lymphoid tissues, such as the mesenteric lymph nodes [16–18]. Dissemination from the distal ileum to the spleen and liver is relatively common, followed by extracellular replication and formation of monoclonal microabscesses [19]. The most common infection is acute gastroenteritis, mainly observed in children and infants on account of being somewhat immunocompromised due to an immature immune system. However, a host of other infections and complications can also occur in older children and adults, including pseudoappendicular syndrome, mycotic aneurysms [20–28], and, more rarely, sepsis as a secondary complication of yersiniosis or from blood transfusions. Several chronic conditions have also been described including: reactive arthritis, erythema nodosum, uveitis, glomerulonephritis, and myocarditis [3, 29]. While enteropathogenic yersiniosis is typically self-limiting in healthy individuals, the mortality rate can reach as high as 50% in immunocompromised persons, as a result of systemic bacterial dissemination [30].

1.2. YPT Infections. YPT causes zoonotic infections in a variety of hosts, including both wild and domestic animals and birds [31]. Human YPT infections, though less common than those caused by YE, are most often acquired from contaminated food or water [32]. Clinically, YPT infections typically present as abscess-forming mesenteric lymphadenitis and diarrhea but can also lead to secondary complications, such as perforation [33], subacute obstruction syndrome [34], intussusceptions [35], and acute renal failure [36] in rare cases. Additionally, patients with severe gastrointestinal bleeding in cases of YPT colitis have also been reported [37–39]. Similar to YE, the most common features of YPT infections in humans are ileocolitis and mesenteric lymphadenitis [40], the latter of which can affect appendix tissue and be mistaken for appendicitis [41]. YPT infections can be acute or chronic [42], with reticulogranulocytic infiltration, enlarged follicles, and necrosis with abscess formation in mesenteric lymph nodes [39, 43, 44]. Infection is usually self-limiting, but rare cases of sepsis can lead to a very high mortality rate (>75%) [45]. In addition to appendicitis, YPT infections have been confused with tumoral lesions [46], terminal ileitis, and Crohn's disease [47]. YPT has also been implicated in reactive arthritis, erythema nodosum, and Kwasaki autoimmune syndrome [48].

1.3. YE Epidemiology. Surveillance of human YPT infections is not routinely performed, and there are thus no complete databases from which information can be used to gauge trends in human YPT infections. However, there are several national surveillance networks that include yersiniosis in weekly, monthly, and yearly reports of human enteric disease cases/isolations, particularly those collected by member states of the European Union, the United States, and New Zealand. Potential sources of epidemiological data include clinical reports, laboratory isolations, sentinel site studies, reported cases, and rates calculated as cases per 100,000 persons in the affected population surveillance area per annum. Differences in reporting methods, isolation methods, and availability of strain information greatly complicate comparisons among countries and sometimes even among different regions/states/territories within an individual country. Furthermore, yersiniosis is infrequently monitored in developing countries, where enteric diseases are a major cause of infant and child mortality. For instance, the World Health Organization initiated a plan to address this issue in Africa in 1998 by working with member states and technical partners to implement the integrated disease surveillance and response (IDSR) program, but yersiniosis is not included as a primary surveillance target. Similarly, the Medical Sciences Center for Disease Control (http://www.moh.gov.cn), a division of China's Ministry of Health, reports communicable disease incidences on a weekly basis, but the plague is the only yersiniae-associated disease included in their surveillance efforts.

Despite the lack of surveillance in many countries, including Africa, Asia, the Middle East, Pacific Islands, Latin America, the Caribbean, and others, there are several national agencies in North America and Europe that provide yearly reports which include sporadic yersiniosis cases, outbreaks, and incidence rates in both humans and animals. As shown in Figure 1, there was a broad range of case reports for North America (including the US and Canada), Oceania (including Australia and New Zealand), and several European countries. For instance, Ireland reported between 3 and 14 isolations of YE/YPT from humans between the years of 2000 and 2009, while Germany reported between 3,906 and 7,186 confirmed cases of human yersiniosis during this same time

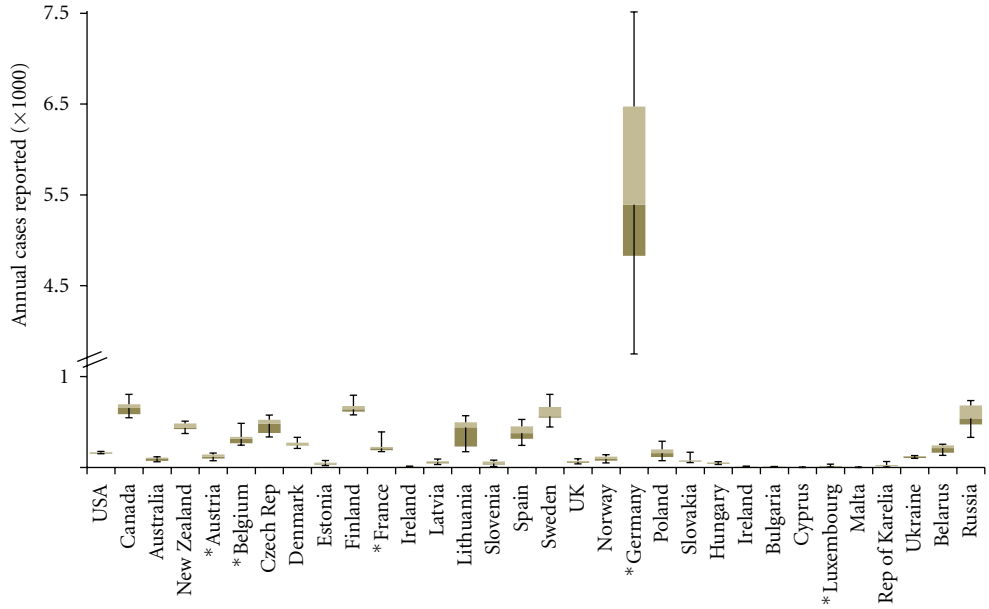

Figure 1: Human yersiniosis cases reported for selected countries that conduct active annual surveillance for the yersiniae. Surveillance data for years 2000 to 2009 were collected from national repositories for Canada (National Microbiology Laboratory, http://www.publichealth.gc.ca), the United States (FoodNet, http://www.cdc.gov/foodnet), 24 European Union members (European Food Safety Authority, http://www.efsa.europa.eu), New Zealand (The Institute of Environmental Science and Research, http://www.surv.esr.cri.nz), Australia (OZFoodNet, http://www.ozfoodnet.gov.au), Northwestern Russia, the Republic of Karelia, Ukraine, and Belarus (EpiNorth Project, http://www.ozfoodnet.gov.au). Russian data was obtained only from the following participating regions: Arkhangelsk oblast, Kaliningrad oblast, Leningrad oblast, Murmansk oblast, Nenets Autonomous okrug, Novgorod oblast, Pskov oblast, St. Petersburg City, Vologda oblast, and the Republic of Komi. For comparison, countries defined as Western European nations based on the classification scheme used by the United Nations include Austria, Belgium, France, Germany, and Luxembourg (which are marked with an asterisk). As shown, Germany reported the greatest number of human cases per annum for the ten-year period included (years 2000–2009), compared to all other countries examined, including the bordering countries of Denmark, Poland, Czech Republic, Austria, and France. The annual cases reported are shown on the *ordinate*, with the axis broken between 1,000 and 4,500 cases to allow the inclusion of Germany and other countries in one graphical display. Yearly cases were not adjusted for population differences. Individual countries are listed on the *abscissa*. USA: United States; UK: United Kingdom.

period (Figure 1). Although, incidences have declined over the last 10 years (Figure 2), German yersiniosis cases account for more than half of all reported European yersiniosis events and ~90% of those within Western European nations that regularly surveyed their populations for YE-associated infections during the aforementioned ten-year-time frame (Figure 1). The reasons for the dramatically higher yersiniosis incidence rate in Germany compared to all other countries with active YE/YPT surveillance programs is unclear, but potential factors include variability in yersiniae isolation procedures and reporting systems, differences in clinical diagnostic frequency, degree of underreporting, prevalence of YE and YPT in animal reservoirs, differences in food processing, and variability in the consumption of meat products. There is some evidence to support the idea that higher meat consumption, particularly pork in Germany compared to other European nations might correlate with Germany's higher incidence of yersiniosis [49].

1.4. YE Genomics. YE is a heterogeneous group of organisms characterized by six biotypes and 60 serotypes. Biotypes can be distinguished based on level of pathogenicity, only one of which is nonpathogenic (Biotype 1A). "Old World" YE includes Biotypes 2–5, which are weakly pathogenic. Most virulent is the "New World" Biotype 1B, which is highly pathogenic to humans and lethal in a mouse model of infection [50]. Of the sixty serotypes of YE, only eleven have been associated with disease in humans, and the majority can be traced to only three commonly virulent serotypes: O:3, O:8, and O:9. These three serotypes are generally considered the causative agents of yersiniosis and vary based on geography. For instance, strain 1B/O:8 has been the predominant version of pathogenic YE in the United States [15]; in contrast, strain 3/O:9 is the most common cause of yersiniosis in China and in Europe [51, 52].

Isolates from these two pathogenic strains were sequenced [53, 54] and recently compared to identify common and unique virulence regions [54]. The results of this analysis indicated that the two strains share considerable genetic conservation/similarity, including most of the known YE virulence determinants. However, several 1B/O:8 key virulence regions were absent in the 3/O:9 strain [54] including high pathogenicity island (HPI) [55], *Yersinia* type II secretion 1 (*yts1*) [56], and the *Yersinia* Type III secretion apparatus (*ysa*). Likewise, the 3/O:9 strain possessed pathogenicity regions absent in the highly pathogenic 1B/O:8 strain. Strain

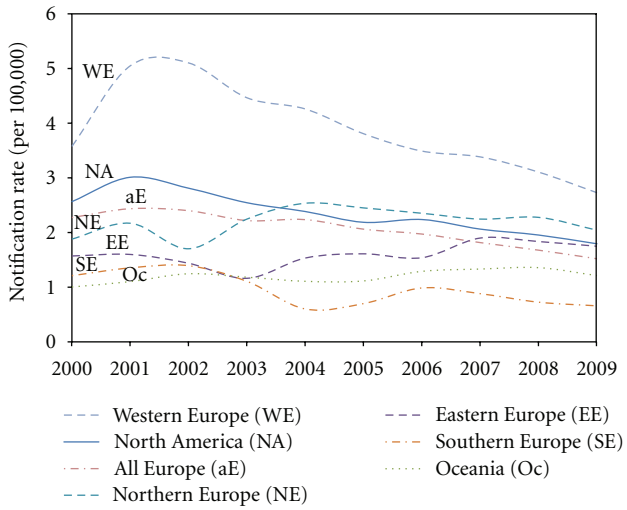

Figure 2: Line graph comparing the yearly incidence rate of yersiniosis reported for various European countries, North America, and Oceania. Surveillance data were collected from national repositories for Canada (National Microbiology Laboratory, http://www.publichealth.gc.ca), the United States (FoodNet, http://www.cdc.gov/foodnet), 24 European Union members (European Food Safety Authority, http://www.efsa.europa.eu), New Zealand (The Institute of Environmental Science and Research, http://www.surv.esr.cri.nz), Australia (OZFoodNet, http://www.ozfoodnet.gov.au), Northwestern Russia, the Republic of Karelia, Ukraine, and Belarus (EpiNorth Project, http://www.epinorth.org). The yearly incidence rate (cases per 100,000 in the surveillance population) was calculated based on total reported cases per year and published population figures included in published surveillance reports or governmental census sites. For countries where surveillance did not include the entire population, rates were adjusted based on the surveillance population and case information provided with the original surveillance data. For countries that did not provide data for all years included in the analysis (i.e., 2000–2009), the rate was extrapolated using linear regression (e.g., Canada, Australia, and Luxembourg). Notification rate (calculated as explained above) per 100,000 persons is shown on the *ordinate*, and a total of 30 countries presented by region are displayed on the *abscissa*. Western Europe (WE) includes Austria, Belgium, France, Germany, and Luxembourg. North America includes Canada and the United States. Northern Europe includes Latvia, Lithuania, Estonia, the United Kingdom, Ireland, Denmark, Norway, Finland, Sweden, and the Republic of Karelia. Eastern Europe includes the Czech Republic, Poland, Slovakia, Hungary, Bulgaria, the Ukraine, Belarus, and Northwestern Russia. Southern Europe includes Slovenia, Spain, and Malta. Oceania includes New Zealand and Australia. All of the available European data considered together (aE), representing a total of 28 countries, is also shown for comparison.

3/O:9-specific regions included a novel chromosomally encoded Type III secretion system (T3SS), ATP binding cassette transporter system, toxin-related gene clusters, and a flagellar gene cluster [54]. Sequencing additional YE strains, such as 4/O:3 that has recently emerged as an important cause of yersiniosis in the United States [57], will likely contribute to a better understanding of the relationship between strain-specific virulence factors and variations in clinical sequelae.

1.5. YPT Genomics. YPT can be classified into 14 distinct biotypes [58], five of which are almost exclusively pathogenic (O1–O5). The remaining nine biotypes (O6–O14) have been isolated from animals and the environment but never from human clinical samples [58–61]. Both pathogenic and non-pathogenic YPT can be further subdivided into 21 serotypes [62] based on the distribution of about 30 different O factors (O-specific polysaccharide of lipopolysaccharide [LPS]) within the species [58]. These serotypes vary geographically and in degree of pathogenicity [63], generally correlating with the size and presence of the chromosomal pathogenicity island, HPI [63]. Only Biotype O1 strains contain a complete, intact HPI. Biotype O3 contains a truncated version, and the pathogenicity island is entirely absent from all other YPT strains that have thus far been examined [64–66]. The pathogenicity of YPT depends on the presence of the T3SS-encoding virulence plasmid pYV [67], YPMa [68], and HPI [69] (described in detail in the next section), and clinical features are closely correlated with the various combinations of these three virulence factors. For instance, pYV is absent in one-fourth of the known virulent serotypes, which instead express the YPMa superantigen variant and/or HPI proteins [63]. The heterogeneous distribution of these factors accounts for the differences in clinical manifestations of infections in the Far East, Europe, and Western countries [63, 66, 70–72].

1.6. YE and YPT Virulence Factors. The genomes of YE, YPT, and YP are 97% identical, but the three bacteria cause vastly different diseases in humans, despite having a shared tropism for lymph nodes [73–76]. Their distributions of shared and unique virulence factors play a critical role in the different routes of infection, types of infections, and severity of disease in humans. Both chromosomal and plasmid-derived virulence factors play a role in yersiniae pathogenesis and in the establishment and progression of yersiniosis. YE pathogenicity depends on the presence of the 70-kb plasmid associated with *Yersinia* virulence, pYV [67, 77–79]. The pYV plasmid differentiates pathogenic from non-pathogenic strains, because it is essential for virulence [79]. The highly pathogenic *Y. enterocolitica* biotype 1B also harbors the chromosomal high-pathogenicity island (HPI), as do almost all European strains of *Y. pseudotuberculosis* serotype O1 [69]. HPI encodes proteins that are involved in the biosynthesis, regulation, and transport of the siderophore yersiniabactin [80, 81] and has thus been referred to as an "iron capture island" [63, 69]. There are five main genes within this island (*psn*, *irp1*, *irp2*, *ybtP*, and *ybtQ*) that are involved in the yersiniabactin system [80, 82, 83]. This system is positively regulated by YtbA, which is, itself, negatively regulated by the iron-responsive regulator Fur [84]. The *psn* and *irp2* genes are important for the high-pathogenicity phenotype of YPT [69, 85].

Almost all Far Eastern strains of YPT additionally produce one of three variants of a chromosomally encoded novel

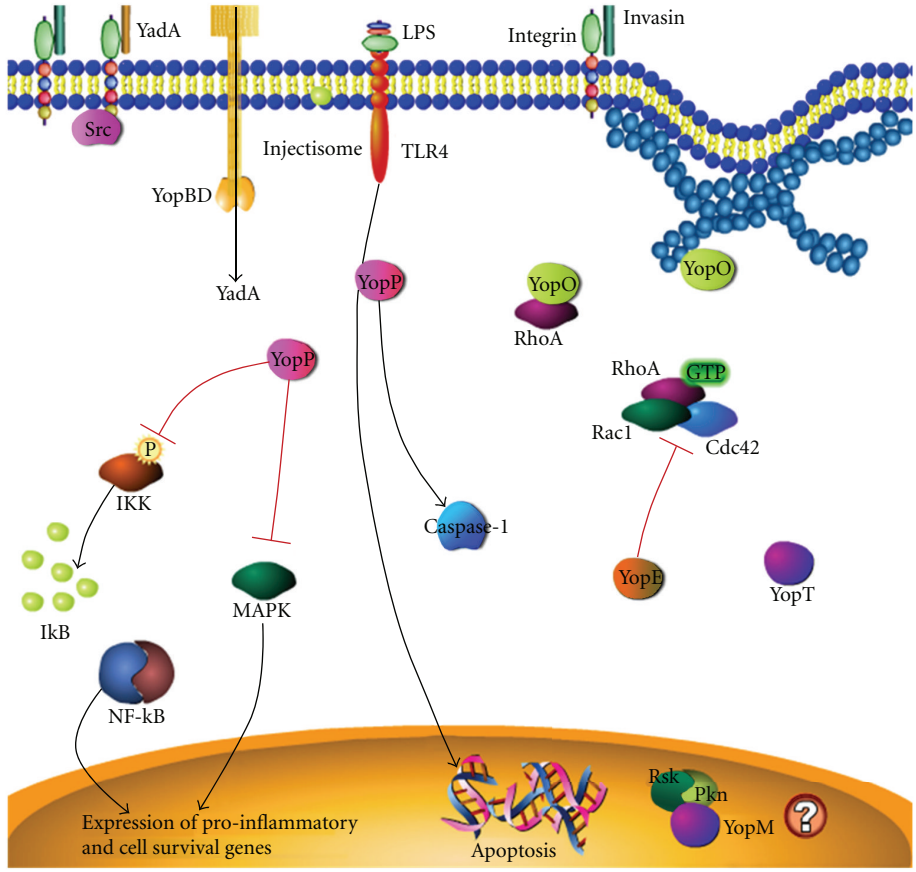

Figure 3: Mechanisms of action of the enteropathogenic yersiniae Ysc T3SS effectors (Yops) on host cell signaling and survival. As shown, membrane-bound *Yersinia* Yad and invasin proteins bind host cell β1-integrins, bringing the bacteria into close proximity to the host cell thereby facilitating insertion of the T3SS injectisome needle-like structure into the targeted host cell. Yops are then translocated across the host plasma membrane and into the cytoplasm, where they interact with the cytoskeleton and host cell signaling molecules. YopO/YpkA interacts directly with the cytoskeleton, as well as the small GTPase signaling molecules, RhoA, Rac1, and Cdc42. YopE inhibits the activities of RhoA, Rac1, and Cdc42. YopP/J promotes LPS-induced host cell apoptosis and directly induces capsase-1 cleavage. YopP/J also inhibits mitogen-activated protein kinases (MAPK) and IKK-mediated NF-κB activation, which prevents expression of proinflammatory and cell survival genes. YopM forms a complex with Rsk and Pkn in the host cell nucleus, which is believed to contribute to bacterial pathogenesis. The figure was produced using Pathway Builder 1.0, a cell signaling drawing tool provided through the Protein Lounge (http://www.ProteinLounge.com).

superantigenic toxin YPM (YPT-derived mitogen) encoded by the *ypm* gene [86, 87]. The original YPM (renamed YPMa) is encoded by *ypmA* [88] and plays a more important role in systemic infections than in gastroenteritis [68]. The other two variants, YPMb and YPMc, are encoded by the *ypmB* and *ypmC* genes, respectively [88, 89].

The small conserved RNA chaperone protein, Hfq is required for full virulence of a variety of pathogenic bacteria, including both YE and YPT [90]. Hfq is required for expression of the heat-stable enterotoxin Yst in YE [91]. In YPT, Hfq plays a role in the regulation of motility, intracellular survival, and production of T3SS effectors [90].

The YPT chromosomally encoded PhoP/Q system [92] regulates survival and growth in macrophages [93, 94] and covalent modifications of LPS that reduce its stimulatory capacity [95], thereby empowering bacteria to avoid, minimize, or delay macrophage activation. In a mouse model of intestinal infection, mutants devoid of PhoP were 100-fold attenuated in virulence due to a reduced capacity to survive and replicate intracellularly within macrophages [93]. The global PhoPQ regulon also senses the reduction in Mg^{2+} and possibly Mn^{2+} levels that characterizes the intracellular environment of host cells. MntH, a putative *Yersinia* Mn^{2+} transporter, was recently proposed to promote survival of the bacteria within phagocytic vacuoles by protecting them from reactive oxygen species [96].

1.7. Establishment of Yersiniosis Infection. In many pathogens, virulence factors are closely coupled to temperature, and this temperature regulation is particularly important for the establishment of infection. At environmental temperatures (less than 28°C) and under acidic conditions at 37°C, the enteric yersiniae optimally express the invasin protein, which is encoded by the chromosomal *inv locus* [17, 18]. Upon ingestion, invasin binds to B1 integrins on host cells and facilitates penetration of the epithelial layer (Figure 3).

The gradual increase in temperature within the host induces the expression of virulence factors necessary to establish a stronghold within the lymph tissues and evade immune system detection. Expression of the chromosomal *ail* (*attachment invasion*) *locus*, for instance, is induced at 37°C, and the resulting Ail/OmpX protein further enhances epithelial cell invasion. Establishment of infection also requires translocation of toxic effectors via a T3SS as well as "other transporter systems" [97]. Regulation of adherence and invasion is mediated via the regulator of virulence A (RovA), which positively regulates *inv* expression, *Yersinia*-modulating protein (YmoA), and histone-like nucleoid structuring protein (H-NS) [98–103].

Yersinia adhesion A protein (YadA) also mediates mucus and epithelial cell attachment and, in concert with invasin, promotes host cell invasion (Figure 3). YadA is a multifunctional, surface-exposed virulence factor encoded on the pYV virulence plasmid that confers the ability to adhere to extracellular matrix proteins [104–106]. Induction of YadA expression is coordinated with the upregulation of Yops (*Yersinia* outer membrane proteins) [107, 108]. The contribution of YadA to virulence is greater for YE than for YPT, playing a significant role in the positive regulation of both adherence to and invasion of host cells [105, 109]. YadA plays only a minor role in YPT, conferring merely an adhesive phenotype [110–112]. Similar to invasin, YadA initiates internalization by binding to extracellular fibronectin that is bound to a 5b1 integrin [105]. YadA from YPT and YE binds fibronectin, collagen I, II, and IV, and laminin, albeit with different affinities thus promoting variable virulence properties [105]. YadA elicits an inflammatory response in epithelial cells by inducing mitogen-activated protein kinase-(MAPK-) dependent interleukin (IL)-8 production and by contributing to the resulting intestinal inflammatory cascade [113, 114]. Interaction of YadA with collagen has been proposed to contribute to chronic yersiniosis infections, such as the development of reactive arthritis [113–116] which has been demonstrated in a rat model [117–119].

In addition to inhibition and invasion of host cells, both Ail and YadA play significant roles in complement resistance and immune evasion. Ail and YadA inhibit the alternative complement pathway by binding regulator factor H and usurping its natural function to prevent lysis of host cells [120–123]. Ail and YadA similarly subvert the classical complement and lectin pathways by binding to C4b-binding protein, thereby promoting the degradation of the C4b complement factor and preventing the formation of the C3 convertase that would otherwise lead to lysis of the bacterial cells [123].

Other YPT virulence factors include the putative DNA adenine methyltransferase, YamA, which is required for full virulence [124], and several proteins that aid in bacterial survival under acidic conditions. An aspartate-dependent acid survival system was recently described for YPT, which plays a role in bacterial survival and thus facilitates establishment of infection [125]. A drop in pH induces the expression of the YPT *aspertase* (*aspA*) gene; the encoded gene product, AspA, subsequently produces ammonia, allowing the ingested organisms to survive the acidic gastrointestinal environment [125]. Other bacterial factors that promote survival under acidic conditions include urease [126], TatC [127], PhoP, OmpR, and PmrA [128, 129]. Acidic pH also induces a downregulation of the transcriptional regulator, Cra (for catabolite repressor/activator), which increases bacterial acid survival [130]. Presumably Cra mediates this action via transcriptional regulation, but its mechanism of action remains unknown.

1.8. T3SS and Yop Effectors. The T3SS, which is encoded on the pYV virulence plasmid and is common to all three pathogenic yersiniae, plays a substantial role in both the establishment and outcome of infection. The T3SS injectisome spans both the inner and outer bacterial membranes, and virulent effector proteins, termed *Yersinia* outer proteins (Yops), are translocated through a host-cell docked *Yersinia* secretion protein F (YscF) needle, directly into the targeted host cells [131]. The YopB and YopD proteins form a pore in the host cell plasma membrane, allowing for the docking of the YscF needle and eventual translocation of the effectors (Figure 3). Proper assembly of a stable injectisome complex also requires the YscE and YscG cytosolic chaperone proteins [132]. There are six effector Yop proteins (YopE, YopH, YopP/J, YopO/YpkA, and YopM) that mediate immune evasion by interfering with host signal transduction pathways, disruption of the host actin cytoskeleton, and by inducing host-cell apoptosis (Figure 3) [133, 134].

Delivery of Yops requires close contact between the bacterial and host cells and is mediated by YadA and invasin through their binding to β1-integrins (Figure 3) [135, 136], which when stimulated cause the activation of Src kinases and RhoA that facilitate Yop translocation via modulation of actin polymerization [137]. In the absence of Yops, activation of β1-integrins would instead lead to actin rearrangements that promote bacterial internalization [138]. Each Yop has a designated chaperone called a Syc protein (for specific Yop chaperone) (e.g., SycE for YopE), required for Yop secretion [133]. The T3SS injectisome is triggered by host-cell contact [139], as well as *in vitro* by temperature (37°C) and low calcium conditions (which serve to emulate intracellular conditions of the host cells) [140–142]. Yop effectors allow evasion of immune responses by blocking host phagocytic function [133, 143, 144], which is vital for bacterial replication and intracellular survival. The *Yersinia* T3SS pore itself was recently suggested to trigger processing of IL-1β and IL-18 in macrophages [75, 145] and subsequent formation of an inflammasome, a cytosolic innate immune complex [146] that triggers inflammation and pyroptosis in response to pore formation [147, 148].

Host cell death is mediated by the YopP/J effector, a serine-threonine acetyltransferase that induces apoptosis of phagocytes by modulating the actions of LPS (Figure 3). Upon binding to the toll-like receptor (TLR)-4, LPS induces the activation of proapoptotic host factors via TRIL (Toll/IL-1 receptor domain-containing adapter inducing IFN-β) [149, 150], while simultaneously downregulating proinflammatory and cell survival genes via inactivation of MAPK and nuclear factor kappa B (NF-κB) transcription factor (Figure 3)

[151–153]. YopP/J specifically inhibits the inflammatory and cell survival actions of LPS [154, 155], thus tipping the scale towards host cell apoptosis [150, 156]. YopP/J-mediated inhibition of host cell proinflammatory responses involves inhibition of IKKβ activation, and thus NF-κB activity (Figure 3) [157], which results in the reduction of TNF-α release by macrophages [158], prevention of IL-8 secretion by epithelial cells [155], and reduction in the presentation of ICAM-1 and E-selectin adhesion factors on the surface of epithelial cells [159]. More recently, it was shown that YopP/J also directly activates caspases (Figure 3) independently of upstream death receptors [160–162].

Once injected into the host-cell cytoplasm, YopE, -H, -P, and -T cooperatively disrupt the cytoskeleton of epithelial cells, macrophages, and dendritic cells thereby decreasing their capacity to engulf the invading bacteria. YopP/J can also facilitate evasion of adaptive immune responses by inhibiting the ability of dendritic cells to present antigens to $CD8^+$ T cells [163], either directly or possibly by decreasing the population of dendritic cells via induction of apoptosis [162, 164, 165]. A similar strategy is employed by YPT using the GTPase activating protein (GAP), YopE, to circumvent phagocytosis by dendritic cells [163, 166]. In addition to the *Yersinia* injectisome and effector proteins, at least three adaptor proteins YopB, YopD, and VirF/LcrV (low calcium response V antigen) are required for T3SS activity [133]. VirF/LcrV (also called V antigen) is a multiple adaptational response (MAR) family member that regulates the T3SS at the level of transcription and, when secreted into the extracellular host environment, contributes to virulence by downregulating inflammation [167, 168].

YopE, YopT, and YopO/YpkA counteract host-cell phagocytosis by acting on monomeric Rho GTPases responsible for regulation of cytoskeleton dynamics [133]. YopE exhibits GAP activity, thereby inducing GTP hydrolysis and, thus, inactivation of RhoA, Rac1, and Cdc42 (Figure 3) [169–171]. YopT, on the other hand, acts as a cysteine protease that inactivates Rho, Rac, and Cdc42 *via* cleavage [172, 173]. YopO/YpkA is a serine-threonine kinase with sequence and structural similarity to RhoA-binding kinases that undergoes autophosphorylation upon binding to actin [174–176]. YopO can also bind directly to RhoA and Rac-1 with currently unknown consequences [133].

The YopH effector was also recently shown to inhibit host inflammatory responses *via* the downregulation of chemokine monocyte chemoattractant protein 1 (MCP-1) [177]. YopH of YPT inhibits activation of the phosphatidylinositol 3-kinase pathway, resulting in the prevention of antigen-mediated activation of lymphocytes [177, 178]. YopH, a protein tyrosine phosphatase, disrupts T-cell and B-cell activation by interfering with phosphorylation signaling events resulting in decreased expression of the costimulatory molecules B7.2 and CD69, as well as the leukocyte mitogen, IL-2 [178, 179]. Very little is known about YopM, but its deletion results in a dramatic decrease in virulence [180]. YopM appears to be injected into host cells, along with other T3SS effector proteins [181], but there is also evidence that YopM can bind to the extracellular acute phase protein α1-antitrypsin [182]. More recently, YopM was shown to form a complex with ribosomal S6 kinase (RSK) and protease-activated kinase (PKN) (Figure 3) [183], which results in sustained activation of RSK and possibly contributes to *Yersinia* pathogenicity [184, 185].

1.9. Chromosomal T3SSs. In addition to the pYV-encoded T3SS, there are two additional chromosomally encoded T3SSs in YE: a flagellar T3SS and the Ysa T3SS [186, 187]. The Ysa T3SS is optimally expressed under high salt concentrations, 26°C, and at stationary growth phase [186, 188, 189]. Salt responsiveness is mediated by the sycBysp BCDA operon, which is regulated by YsaE and the SycB chaperone [189]. The Ysa T3SS plays a role in virulence [186] and is important for colonization of the small intestine despite its optimal expression at non mammalian temperatures (26°C) [190]. There are 15 known Ysa effector proteins (Ysps), which are thought to function similarly to Yop effectors as modulators of host immune responses [191]. Interestingly, the flagellar T3SS, which functions in the biogenesis of flagella, secretes Fop effectors that also play a role in the pathogenesis of YE [187]. YplA (*Yersinia* phospholipase A), for instance, is a Fop required for colonization of Peyer's Patches and mesenteric lymph nodes that contributes to inflammatory responses within these tissues [192].

1.10. Type VI and IV Secretion System. T3SSs are not the sole secretion systems identified in the yersiniae that promote bacterial virulence. In fact, a type VI secretion system (T6SS) was recently identified in YPT, which harbors four copies, one of which was recently shown to be regulated by temperature, growth phase, and the N-acyl homeserine lactone-AHL-dependent quorum sensing system [193]. YPT also harbors a type IV pilus gene cluster that contributes to pathogenicity [194].

1.11. Host Responses to YE and YPT Infection. *Yersinia* infections are biphasic and are initiated by a "quiet" 36–48 hour period of bacterial replication without a measurable host response. This initial "quiet" phase is followed by an influx of activated phagocytes into infected tissues and lymph nodes, which induces an acute inflammatory response characterized by cytokine production and tissue necrosis [74, 76, 195–199]. The T3SS Yop effectors are likely responsible for the initial inhibition of phagocytic functions, but the mechanisms behind such a sudden, bipolar "off-on" inflammatory response are presently not fully understood. The T3SS is absolutely required for effective colonization of systemic organs, and T3SS inactivation leads to rapid clearance of the bacteria by the host [200–202]. As a result, yersiniae lacking a functional T3SS are avirulent and can function as live attenuated vaccine strains in mice [200, 203, 204].

Recent evidence suggests that macrophages can compensate for YopE/YopH-mediated inhibition of the endosomal MHC class II antigen presentation pathway by an autophagy-dependent mechanism [205]. Thus, autophagy might serve as an alternative counter-pathway by which the host might mount an MHC class II-restricted $CD4^+$ T-cell response

against *Yersinia* T3SS-mediated translocation of Yop virulence effectors [205]. However, whereas Deuretzbacher et al. [206] demonstrated autophagy-mediated degradation of macrophage internalized YE, YPT was shown to usurp the autophagosome pathway for continued replication within macrophages at the intestinal site of infection [207].

Murine studies have demonstrated that $CD4^+$ and $CD8^+$ T cells are required for control of YE infection [196, 208], as are IFN-γ-mediated Th1 immune responses, including macrophage production of TNF, IL-12, and IL-18 [209–212]. Inhibition of T-cell proliferation and dendritic cell functions by Yops are primary mechanisms by which the yersiniae evade both innate and adaptive immune responses [213]. Interestingly, the yersiniae induce both apoptosis of naïve macrophages and inflammatory cell death (pyroptosis) of activated macrophages, which is consistent with its biphasic infection process [73, 75]. Increased inflammation associated with the redirected host cell death could initially benefit the yersiniae but later could contribute to a generalized immune response and eventual clearance of bacteria [73, 75].

1.12. Detection and Prevention of Food-Borne Yersiniosis. YE and YPT clinical infections most often occur following ingestion of the bacteria in contaminated food or water. The two aforementioned yersiniae have been isolated from meat, fresh produce, and milk, but their presence is frequently unapparent due to detection difficulties. Various YE strains are most often distinguished by pulsed-field gel electrophoresis (PFGE), but there is currently no standardized test or database for consistent identification. Moreover, enteropathogenic *Yersinia* species are not included in the protocols that are used by laboratories in PulseNet which, in cooperation with the Association of Public Health Laboratories (APHL), coordinates with public health laboratories to subtype bacterial foodborne pathogens [214]. The heterogeneity of both YE and YPT makes definitive detection difficult, and PFGE produces multiple bands that are not especially distinctive based on serotype [29, 215–217]. Some reports have suggested that current detection methods can produce false-negatives or false-positives based on variability in the presence of *Yersinia* virulence factors, and their variable correlation with pathogenicity [218, 219]. Suggestions for improving detection include the use of more than one restriction nuclease in PFGE analyses [29] and application of a recently developed multilocus variable-number tandem-repeat analysis (MLVA) for YE [220, 221].

Detection is an especially important concern, because both YE and YPT can readily proliferate at refrigeration temperatures (4°C) and even as low as 0°C. Furthermore, the enteropathogenic yersiniae can likewise adapt to and thrive under modified atmospheric conditions that are often used in conjunction with colder temperatures as common methods of food preservation. Survival and cell growth at low temperatures are accomplished via a short-term, cold-shock response, in which a variety of stress response proteins are produced that mediate bacterial adaptation to the sudden drop in temperature (reviewed in [222]). Both YE and YPT are also capable of more long-term cold adaptation, a process that requires polynucleotide phosphorylase (PNPase), a cold-shock exoribonuclease that enhances both T3SS function as well as promoting growth under cold conditions [223].

Pathogenic YE produce insecticidal toxins, encoded by *tc* (*toxin complex-like*) genes located within a chromosomal pathogenicity island [224, 225]. These insecticidal toxins are expressed at low temperatures [226], but they are nonetheless thought to possess virulence functions in mammalian hosts [224, 225]. It is possible that the presence of these insecticide toxins suggests that the normal life cycle of YE includes an insect stage, as previously proposed [226], and these toxins might facilitate growth of the organisms in refrigerated food products. Tc proteins in YPT, on the other hand, do not possess insecticide activity but rather confer toxicity to mammalian cells [227] and might, therefore, play a role in human disease.

The presence of β-lactamases that confer antibiotic resistance to some pathogenic strains of YE [228, 229] underscores the importance of surveillance for these pathogenic organisms. While these organisms are not monitored nationally, yersiniosis incidence rates and patient demographics in the United States are collected annually by the Foodborne Diseases Active Surveillance Network (FoodNet). FoodNet reported 1,355 and 18 human yersiniosis cases of YE and YPT, respectively, in the U.S. between 1996 and 2007. However, based on FoodNet's assessments [230], cases of yersiniosis, especially those caused by YPT, are likely under-estimated in the U.S. due to lack of testing and difficulty associated with culturing the yersiniae on standard media [231, 232].

2. Conclusions

YE is the major cause of yersiniosis in humans, although prevalence of YPT-associated disease is likely underreported due to lack of surveillance and differences in applied isolation strategies. Extreme heterogeneity among strains of YE and YPT further complicates efforts to link contamination to the source and monitor human disease in a uniform manner comparable to other more thoroughly studied food-borne pathogens (e.g., *Salmonella*). Although a plethora of animal hosts serve as reservoirs for both YE and YPT, human disease-associated yersiniae are most prevalent in swine. In healthy individuals, the resulting illness can manifest as mild, self-limiting diarrhea, but in young children and immunocompromised individuals yersiniosis can represent a significant source of morbidity and mortality. Additionally, chronic diseases, such as reactive arthritis and secondary (or nosocomially derived) complications such as sepsis, can develop in immune compromised persons.

YE and YPT are heterogeneous organisms that differ in genomic content and degree of pathogenicity. Two pathogenic strains (1B/O:8 and 3/O:9) have been sequenced and compared [53, 54] to gain insight into virulence mechanisms required to initiate infection and cause acute symptoms or chronic conditions in patients. YE infection is generally established via consumption of contaminated food or water and involves adherence to and translocation across the intestinal

barrier via M cells [16]. Other virulence factors include the pYV plasmid, which encodes a T3SS essential for YE pathogenicity [79], and the chromosomal HPI locus found in highly pathogenic strains [69]. Pathogenic YPT strains encode a novel superantigenic toxin, YPM that contributes to systemic infections [68] and a PhoP/Q system important for regulation of bacterial survival and growth within macrophages [93, 94]. Type IV pilus genes [194] and a recently discovered T6SS [193] also contribute to yersiniae virulence. While a great deal of molecular work has contributed significantly to a better understanding of YE and YPT pathogenicity, there is much to be gained from future studies, particularly those aimed at dissecting the contributions of various virulence factor combinations to pathogenicity, the resulting type of infection, and ability of the host immune system to clear the bacteria. Very little is known about yersiniae-associated autoimmune disease and other chronic conditions. For instance, YPT is much less studied than YE and thus might be underappreciated as a causative agent of yersiniosis. As such, yersiniosis surveillance efforts concentrate almost exclusively on YE, making attempts to accurately estimate YPT-associated gastroenteritis incidence nearly impossible.

Enteropathogenic YE and YPT cause yersiniosis globally and are of significant concern to the pork industry. The ability of the enteropathogenic yersiniae to replicate and thrive at refrigeration temperatures, coupled with their seemingly ubiquitous nature, suggests that future and more uniform surveillance measures are inevitable and requisite. At present, enteropathogenic yersiniae cases are likely underestimated; however, recent preventative measures in the pork industry and increased attention, both in the research laboratories and clinics, will provide much needed insight and better strategies for managing yersiniosis. Furthermore, more thorough and uniform surveillance measures will allow us to more accurately gauge national and global yersiniosis trends and better predict which agricultural, hygienic, and clinical efforts are effective in reducing the incidence of yersiniosis infection in the general population.

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Review Article

Yersinia enterocolitica: Mode of Transmission, Molecular Insights of Virulence, and Pathogenesis of Infection

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Although *Yersinia enterocolitica* is usually transmitted through contaminated food and untreated water, occasional transmission such as human-to-human, animal-to-human and blood transfusion associated transmission have also identified in human disease. Of the six *Y. enterocolitica* biotypes, the virulence of the pathogenic biotypes, namely, 1B and 2–5 is attributed to the presence of a highly conserved 70-kb virulence plasmid, termed pYV/pCD and certain chromosomal genes. Some biotype 1A strains, despite lacking virulence plasmid (pYV) and traditional chromosomal virulence genes, are isolated frequently from humans with gastrointestinal diseases similar to that produced by isolates belonging known pathogenic biotypes. *Y. enterocolitica* pathogenic biotypes have evolved two major properties: the ability to penetrate the intestinal wall, which is thought to be controlled by plasmid genes, and the production of heat-stable enterotoxin, which is controlled by chromosomal genes.

1. Introduction

Yersinia enterocolitica was discovered more than 60 years ago [1] but was not considered as a human or veterinary pathogen until the late 1960s when it became increasingly identified in foodborne gastrointestinal infections [2, 3]. *Y. enterocolitica* is a member of the genus *Yersinia* which encompasses a heterogeneous collection of facultatively anaerobic bacteria that belong to the family Enterobacteriaceae. Of the 11 species within this genus [4], only three, *Y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica* are regarded as pathogenic for humans whereas *Y. ruckeri* is a fish pathogen, and *Y. enterocolitica*-like organisms *Y. krirtensenii, Y. intermedia, Y. mollaretii, Y. frederiksenii* and *Y. bercovieri* have yet an unidentified role in human disease [5]. *Y. enterocolitica* is associated with a wide range of clinical and immunological manifestations, responsible for intestinal diseases, including enterocolitis with an inflammatory diarrhea in affected infants and young children; acute terminal ileitis and mesenteric lymphadenitis mimicking appendicitis in older children and young adults, as well as rare extraintestinal manifestations including urinary tract and respiratory tract infection (empyema), osteoarticular infection (reactive arthritis), erythema nodosum, infected mycotic aneurysm [6–8], axillary abscesses [9], and endocarditis [10].

The geographical distribution of *Y. enterocolitica* is diverse. *Y. enterocolitica* has more than 50 distinct serotypes (on the basis of antigenic variations in cell wall lipopolysaccharide), and few of them are pathogenic. O:8 is the primary infectious serotype in the USA followed by O:3, O:5, 27, O:13a,13b, O:20, O:9, and so forth [6, 7]. In China, serotype O:3 is primarily found in infections followed by O:9 and O:8 [14]. Furthermore, various serotypes demonstrate geographical specificity; for example, the predominant serotype in Australia, Europe, and Canada is O:3 [5], O:8 in Japan [15], and O:9 in Scandinavia, the Netherlands [16].

The incidence of *Y. enterocolitica* foodborne infection varies according to geography and climate variation. In developed countries, the incidence is higher in infants and

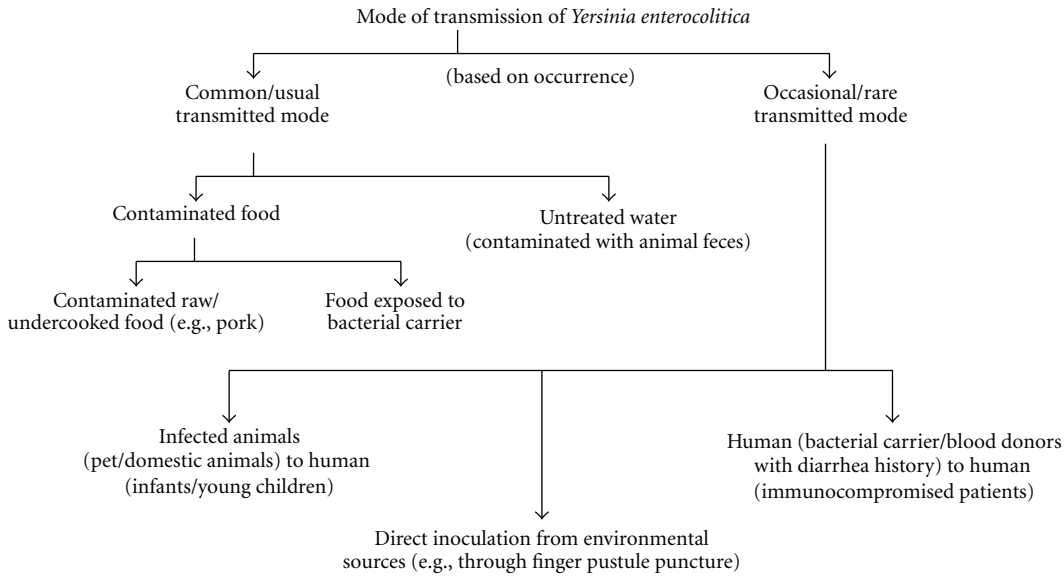

Figure 1: Mode of transmission of *Y. enterocolitica.*

young children, although all ages are at risk. The majority of foodborne infections are sporadic, and the infection sources are unknown, but large outbreaks have also occurred [5, 17, 18]. *Y. enterocolitica* foodborne outbreaks have occurred in Australia, Finland, Japan, Norway, the United States, and Brazil. There were two foodborne outbreaks in China in 1980s; one was caused by beef contamination in Lanzhou of Gansu Province in 1986 with 109 patients with diarrhea caused by *Y. enterocolitica* O:3 infection [19]. The second occurred in a school in Shenyang of Liaoning Province with 352 students having diarrhea caused by *Y. enterocolitica* O:8 infection [14]. Recently, *Y. enterocolitica* has become of concern worldwide, and foodborne infections have been reported in hundreds of countries.

2. Mode of Transmission

2.1. Foodborne Transmission. *Y. enterocolitica* is an important foodborne human enteropathogen that causes sporadic illness and occasional foodborne outbreaks in the United States whereas incidence of yersiniosis and outbreaks appeared to be higher in many European countries than the United States [6, 20]. It has been isolated from many foods, including beef, pork, liquid eggs, soft cheese, raw milk, pasteurized milk, fish, raw oysters, shrimps, crabs, chocolate milk, turkey, chow mein (chop suey served with fried noodles), powdered milk, bean sprouts (especially mung beans, lentils, or edible soybeans), and tofu (cheese-like food made of curdled soybean milk). Although the organism has been isolated from many foods, there have been relatively few foodborne outbreaks attributed to *Y. enterocolitica* in developed countries, for example, Japan and the Netherlands [15, 16] as well as in developing countries, for example, Bangladesh and Iraq [21, 22]. Human yersiniosis is primarily acquired through the gastrointestinal tract as a result of ingestion of contaminated foods—usually raw or inadequately cooked pork [16]. *Y. enterocolitica* foodborne outbreaks in the United States have involved young children exposed indirectly during the cleaning and preparation of raw or undercooked pork chitterlings [23]. Chitterlings are generally well cooked, so it is believed that hands, kitchen surfaces, or other kitchen articles contaminated during the preparation of chitterlings are the vehicles for foodborne infection. Survival of *Yersinia* on these vehicles is facilitated by the hardiness of *Yersinia*, which is able to multiply in adverse conditions like commercial refrigeration temperatures. Other foodborne outbreaks have been associated with untreated water, contaminated tofu, contaminated bean sprouts, and contaminated milk (unpasteurized or inadequately pasteurized milk) [20]. The isolation of *Yersinia* strains from contaminated milk can be probably the result of postpasteurization contamination, since even the most heat-resistant strains are reported to be killed by pasteurization (Figure 1).

2.2. Human-to-Human Transmission. Person-to-person transmission is rare. However, contamination of food by infected food handler and nosocomial infections have been reported. In July 2006, person-to-person transmission was observed in a familial outbreak of *Y. enterocolitica* bioserotype 2/O:9 in Japan [24]. The possible source of this infection was an infected carrier who suffered from diarrhea [24]. In addition, the outbreak of diarrheal disease due to *Yersinia enterocolitica* bioserotype 1/0:5 was reported in hospitalized patients, which was the indication of a nosocomial outbreak due to *Yersinia enterocolitica* [25].

2.3. Animal-to-Human Transmission and Waterborne Transmission. Occasionally *Y. enterocolitica* infection occurs after direct or indirect contact with infected animals. It has been isolated from the intestinal tracts and feces of many animals, including rodents (rabbits), domestic animals (e.g., sheep, cattle, cats, pigs, and dogs) [26], and other animals

(deer, raccoons, and horses) and water contaminated by those animals. The pig appears to be the main reservoir for the strains causing infection in humans. Pig feces are a potential mode of direct transmission to farmers [27]. As *Y. enterocolitica* possess the ability to grow under extreme environmental condition, they are welladapted to survival in cooler temperate zones as well as in microaerophilic environments including aquatic environments.

2.4. Direct Transmission. *Y. enterocolitica* rarely causes extraintestinal disease. In case of extraintestinal disease, direct transmission is proposed as the mode of transmission of this classically enteric pathogen [9]. In January 2009, a 54-year-old African American construction worker with chronic hepatitis C developed an axillary abscess due to *Y. enterocolitica* that followed an injury to his finger. It was proposed that the finger pustule arising as a consequence of traumatic puncture presented the possibility that direct inoculation of *Y. enterocolitica* from an environmental source may have been the mode of transmission. These suggest an alternative nonfoodborne route for *Y. enterocolitica* transmission. A similar route of transmission was proposed for a patient with *Y. enterocolitica* axillary abscess whose employment as a butcher subjected him to frequent cut wounds to the hand [28].

2.5. Blood Transfusion-Associated Transmission. *Yersinia enterocolitica* can be transmitted through contaminated blood, and it was one of the first recognized causes of posttransfusion sepsis [29]. This first case of transfusion-associated sepsis caused by *Y. enterocolitica* was described in the Netherlands in 1975. Since then, more than 60 additional cases have been reported in the literature worldwide. *Y. enterocolitica* has occurred occasionally in donor blood from healthy donors or donors with a diarrhea history; such contaminated blood sometimes caused *Yersinia* bacteremia and death of the recipients [30]. Although fatality due to posttransfusion bacterial-associated sepsis is rare [31], blood-transfusion-associated septicemia due to *Y. enterocolitica* is reported to have high fatality rate. In 2003, a fatal case of septic shock was observed in a 71-year-old patient following transfusion of contaminated red blood cells (RBCs) for refractory anemia. *Y. enterocolitica* bioserotype 4/O:3 was isolated from the patient's blood sample and the transfused RBCs. High titers of antibodies against *Y. enterocolitica* were detected in the donor's plasma sample one month after blood donation. The donor reported abdominal discomfort 3.5 months before blood collection but had no clinical signs of intestinal infection at the time of donation [32].

3. Molecular Insights in Virulence

Yersinia enterocolitica has evolved into an apparently heterogeneous collection of organisms encompassing six biotypes differentiated by physiochemical and biochemical tests (1A, 1B, 2, 3, 4, and 5; Table 1) and more than 50 serotypes differentiated by antigenic variation in cell wall lipopolysaccharide. Of the six biotypes, biotype 1A is the most heterogeneous, and encompasses a wide range of serotypes (Table 2), of which serotypes O:5, O:6,30, O:6,31, O:7,8, O:10, as well as O-nontypable strains, are isolated most often [33]. The virulence of the pathogenic biotypes, namely, 1B and 2–5 is attributed to the presence of a highly conserved 70-kb virulence plasmid, termed pYV/pCD and certain chromosomal genes [42] (Table 3). The biotype 1A strains of *Y. enterocolitica*, on the other hand, have been reported to lack pYV plasmid which encodes virulence factors including *Yersinia* adhesin A (YadA) and Ysc-Yop type III secretion system (TTSS) as well as chromosomally borne virulence genes including *ail, myfA, ystA, ysa*, and the high pathogenicity island- (HPI-) associated iron acquisition system [35] (Figure 2).

3.1. Virulence Factors of pYV-Bearing Strains of Y. enterocolitica [33]. Apart from pYV itself, pYV-bearing strains of *Y. enterocolitica* require a number of chromosomally borne genes to express full virulence. Some of these virulence genes are restricted to pYV-bearing bacteria whereas others occur more widely. Virulence genes that are mostly limited to pYV-bearing strains of *Y. enterocolitica* include *inv* (encodes invasin, an outer membrane protein that is required for efficient translocation of bacteria across the intestinal epithelium) [43]); *ail* (encodes another outer membrane protein that may contribute to adhesion, invasion, and resistance to complement-mediated lysis) [44]; *yst* (encodes *Yersinia* stable heat-stable enterotoxin that may contribute to the pathogenesis of diarrhea associated with acute yersiniosis) [45, 46]; *myf* (encodes a fimbrial antigen and putative adhesin) [47]. In addition, strains of biotype 1B, which are particularly virulent for humans and laboratory animals, carry a high-pathogenicity island (HPI) which facilitates the uptake and utilization of iron by bacterial cells, and hence may promote their growth under iron-limiting conditions in host tissues [48]. Virulence-associated determinants of pYV-bearing *Y. enterocolitica* that also occur in pYV-negative strains include cell surface lipopolysaccharide and SodA (a superoxide dismutase), which appear to facilitate bacterial survival in tissues [49, 50], as well as urease, which enhances bacterial resistance to stomach acid and may also play a role in nitrogen assimilation [51].

pYV functions mainly as an antihost plasmid that permits the bacteria which carry it to resist to phagocytosis and complement-mediated lysis, thus allowing them to proliferate extracellularly in tissues. The pYV plasmid-encoded virulence factors include an outer membrane protein adhesin, YadA, and a type III protein secretory apparatus which translocates effector proteins, known as Ysc-Yops, from the bacterial cell to the cytoplasm of susceptible host cells [42]. The contribution of pYV-encoded factors, in particular YadA and the Yop effectors, to bacterial virulence has been established in a large number of studies. Strains of *Yersinia* which lack pYV are susceptible to killing by complement and polymorphonuclear leukocytes, although they are able to persist in macrophages and nonprofessional phagocytic cells, and cause short-lived infections which are typically asymptomatic [52].

Table 1: Biotyping scheme of *Y. enterocolitica* (adapted from [33, 34]).

Test	Reaction of biotype					
	1A	1B	2	3	4	5
Lipase (Tween hydrolysis)	+	+	−	−	−	−
Aesculin hydrolysis	V	−	−	−	−	−
Indole production	+	+	(+)	−	−	−
D-Xylose fermentation	+	+	+	+	−	v
Voges-Proskauer reaction	+	+	+	+	+	(+)
Trehalose fermentation	+	+	+	+	+	−
Nitrate reduction	+	+	+	+	+	−
Pyrazinamidase	+	−	−	−	−	−
B-D-Glucosidase	+	−	−	−	−	−
Proline peptidase	v	−	−	−	−	−

+, positive; (+), delayed positive; –, negative; v, variable reactions.

Table 2: Relationship between biotype, O serotype, and pYV carriage of *Y. enterocolitica* (adapted from Sharon et al. 2003).

Biotype	Serotype(s)
1A	O:4; O:5; O:6,30; O6,31; O:7,8; O:7,13; O:10; O:14; O:16; O:21; O:22; O:25; O:37; O:41,42; O:46; O:47; O:57; NT[a]
1B	O:4,32[b]; O:8[b]; O:13a,13b[b]; O:16; O:18[b]; O:20[b]; O:21[b]; O:25; O:41,42; NT
2	O:5,27[b]; O:9[b]; O:27
3	O:1,2,3[b]; O:3[b]; O:5,27[b]
4	O:3[b]
5	O:2,3[b]

[a]NT, not typable.
[b]Serotypes which include strains that carry pYV.

Table 3: Virulence-associated genes in *Y. enterocolitica*.

Genes	Gene product/function	Reference
Inv^{C,tr}	Invasin (an outer membrane protein that is required for efficient translocation of bacteria across the intestinal epithelium)	[35]
ail^{C,tr}	Adhesin (outer membrane protein that may contribute to adhesion, invasion, and resistance to complement-mediated lysis)	[36]
virF^{C,tr}	Transcriptional activator	[36]
myfA^{C}	Mucoid *Yersinia* factor (fimbrial antigen and putative adhesin)	[37]
ystA^{C,tr}	Enterotoxin (*Yersinia* stable heat-stable toxin that may contribute to the pathogenesis of diarrhea)	[38]
ystB^{C}	Enterotoxin (*Yersinia* stable heat-stable toxin that may contribute to the pathogenesis of diarrhea)	[38]
ystC^{C}	Enterotoxin (*Yersinia* stable heat-stable toxin that may contribute to the pathogenesis of diarrhea)	[39]
fepA	Enterochelin receptor protein	[40]
fedD	Enterochelin receptor protein	[40]
Fes	Enterochelin esterase	[40]
tccC^{P}	Insecticidal toxin-like protease	[35]
ymoA	*Yersinia* modulator	[41]
hreP	Subtilisin/kexin-like protease (host responsive element)	[35]
Sat	Streptogramin acetyltranferase	[35]
yadA^{P,tr}	*Yersinia* adhesin A	[42]
ysa^{P}	*Yesinia* secretion apparatus	[42]

^{C}*Chromosome borne gene,* ^{P}*plasmid-borne gene,* ^{tr}*traditional virulence gene.*

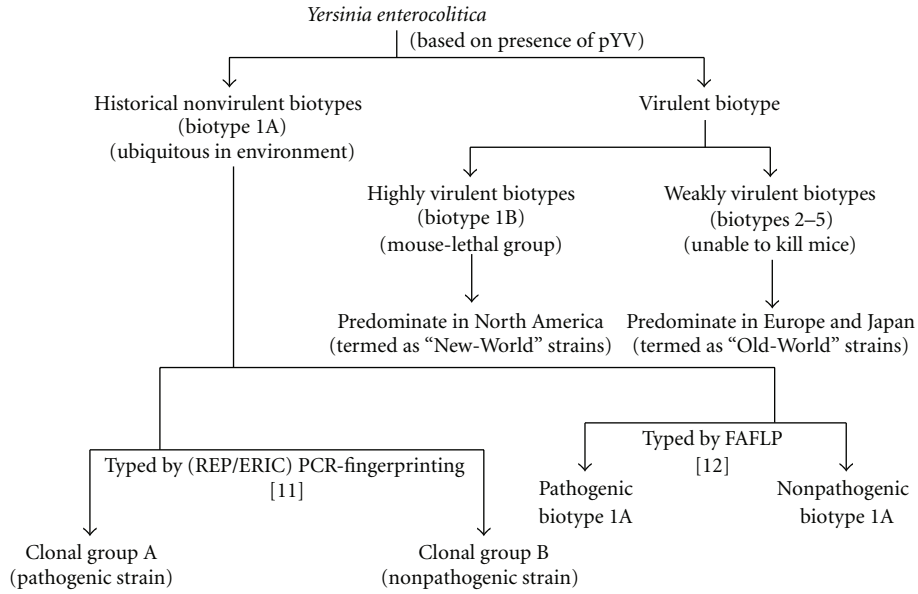

Figure 2: *Y. enterocolitica* biotypes. *Y. enterocolitica* biotypes are classified into three distinct group: a historically defined nonpathogenic group (biogroup 1A); a weakly pathogenic group that are unable to kill mice (biogroups 2 to 5); a highly pathogenic, mouse-lethal group (biogroup 1B). Biotype 1A strains are clustered into two clonal groups (A and B) when typed by repetitive extragenic palindrome (REP)—and enterobacterial repetitive intergenic consensus (ERIC)—PCR fingerprinting [11], and two groups when typed by fluorescent amplified fragment length polymorphism (FAFLP) [12].

3.2. Evidence Indicating the Lack of Virulence of Biotype 1A Strains. Biotype 1A strains of *Y. enterocolitica* are often considered to be nonpathogenic primarily because they do not possess the virulence-associated factors of pYV-bearing strains. The biotype 1A strains have been reported to lack both pYV plasmid and most chromosomal virulence genes such as *ail, myfA, ystA, ysa,* TTSS, and HPI, and only occasionally carry *ystA* and *myfA* [53]. Although the *ail* gene is present in some biotype 1A strains, the *ail* gene alone is an insufficient virulence marker for detecting the virulence of *Y. enterocolitica* biotype 1A strains [54]. Another line of evidence that is taken to indicate the avirulence of biotype 1A strains is their relatively high prevalence in the environment and healthy animals. Indeed, biotype 1A strains are ubiquitous, inhabiting a wide variety of environmental niches such as soil and various sources of water, including streams, lakes, water wells, and wastewater [55, 56] Sharon et al. 2003. They are also frequently isolated from foods, including various vegetables and animal products, such as pork, poultry, packaged meat, seafood, raw milk, and pasteurized dairy products. Biotype1A are also found in a vast array of animals, including birds, fish, various insects, frogs, and a wide range of mammals, including cattle, sheep, pigs, and rodents. In most cases, animals infected with biotype 1A strains are asymptomatic, thus giving support to the concept that these bacteria are avirulent commensals [33] (Table 4).

3.3. Some Studies Indicating the Pathogenicity in Some Y. enterocolitica Biotype 1A Strains. Despite the lack of traditional chromosomal-borne and plasmid-borne virulence genes in *Y. enterocolitica* strains of biotype 1A, some biotype 1A strains are isolated frequently from humans with gastrointestinal diseases. The biotype 1A strains isolated from humans and from pigs have been reported to produce *ystB-encoding Yersinia* heat-stable enterotoxin [53]. A recent study on 259 isolates of *Y. enterocolitica* and related species; indicated that Yst-B (*ystB*) was the major contributor to diarrhea produced by biotype 1A strains of *Y. enterocolitica* [62]. Some biotype 1A strains produce symptoms indistinguishable from that produced by isolates belonging to pathogenic biotypes [63, 64]. Biotype 1A strains have also been implicated in nosocomial [25] and foodborne [65] outbreaks, and were also isolated from extraintestinal infections [66].

4. Pathogenesis

Yersinia enterocolitica pathogenesis is incompletely understood. Most isolates of *Y. enterocolitica* from food or clinical materials have either of two pathogenic properties. First property is the ability to penetrate the intestinal wall, which is thought to be controlled by 70-kb virulence plasmid (pYV/pCD) genes; that is absent in avirulent strains; second one is the production of heat-stable enterotoxin which is controlled by chromosomal genes (*ystA, ystB,* and *ystC*) [61].

4.1. Adaptation. As contaminated foods are considered as the common mode of transmission, this microorganism must first adapt its surface antigenic structures like outer membrane proteins to colonize in the intestines of humans

Table 4: Studies indicating the lack of virulence of biotype 1A strains.

Research studies	References
(1) Two large studies in Belgium, involving the microbiological investigation of more than 24,000 fecal samples over a period of almost 16 years, revealed that infection with biotype 1A was not associated with gastrointestinal symptoms and that biotype 1A strains were more frequent amongst subjects having no gastrointestinal complaints.	Van Noyen et al. [57, 58]
(2) Rabbits were infected perorally with different biotype 1A strains from raw fish (serotype O:6,30) and pig intestine (serotype O:5), respectively, and concluded that these bacteria were avirulent.	Pai et al. [59]Une [60]
(3) Robins-Browne et al. reported that gnotobiotic piglets, inoculated perorally with a biotype 1A strain of serotype O:5, which was originally isolated from milk, rapidly cleared the bacteria without developing any clinical or pathological evidence of disease.	Robins-Browne et al. [61]

at a temperature of about 37°C. This is usually achieved in part through the presence of 70-kb virulence plasmid (pYV). Genes on this plasmid encode for several outer membrane proteins (polypeptides) that are expressed at 37°C but not at 25°C [6].

4.2. Adhesion. Attachment of pYV-bearing strains (pathogenic biotypes 1B and 2–5) of *Y. enterocolitica* to tissue culture cells like HeLa cells or HEp-2 cells cultures has been frequently identified in pathogenic *Yersinia* isolates [5, 67, 68]. However, the ability to produce disease does not correlate with HeLa cell attachment as plasmid cured avirulent strains retain the ability to attach to HeLa cells [69]. When the pYV plasmid-containing strain was grown at 26°C in calcium-containing medium, the bacteria adhered to HeLa cells and HEp-2 cell cultures to a high degree. In contrast, when this strain was incubated at 37°C in the same calcium-containing medium, it attached to the HeLa cells and HEp-2 cell cultures at a reduced level [70]. By insertional inactivation of genes located on the virulence plasmid (pYV), Kapperud et al. [71] identified four plasmid-dependent and temperature-inducible properties related to the bacterial surface properties involved in fimbrial adhesion: (i) a fimbrial matrix covering the outer membrane, (ii) outer membrane protein, YOP1 which is a structural component of the fimbriae, (iii) spontaneous autoagglutination, which is related to the fimbriae, and (iv) mannose-resistant hemagglutination of guinea pig erythrocytes [71].

Although the biotype 1A strains of *Y. enterocolitica* have been reported to lack pYV plasmid, various forms of fimbriae are observed in this biotype. One of fimbriae, designated MR/Y-HA is 8 nm in diameter, agglutinates erythrocytes of 10 different animal species in the presence of mannose and is expressed in vitro at low temperature, but not at 37°C [72]. A second type of fimbriae, designated MR/K-like HA, is 4 nm in diameter and mediates mannose-resistant hemagglutination of chicken erythrocytes, but not erythrocytes from a variety of other species [72]. Expression of these fimbriae in vitro occurs only after serial passages of bacteria for at least 7 days. Moreover, as they do not mediate adherence of bacteria to cultured epithelial cells [73], their contribution to the pathogenesis of infection with biotype 1A strains is unknown [33].

Some strains of *Y. enterocolitica* produce a fimbrial adhesin, named Myf (for mucoid *Yersinia* fibrillae), because it bestows a mucoid appearance on bacterial colonies which express it. Myf are narrow flexible fimbriae which resemble CS3, an essential colonization factor of some human clinical strains of enterotoxigenic *Escherichia coli* [33]. However, *myf* genes-associated virulence of these bacteria is unknown.

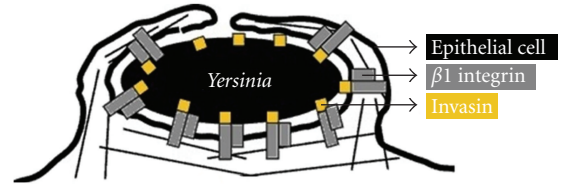

Figure 3: A paradigm of "zippering" entry of a bacterial pathogen into epithelial cells. Invasin mediated binding of Yersinia to β1 integrins and internalization (adapted from [13]).

4.3. Invasiveness (Mechanisms of Epithelial Cell Invasion). Entry of enteroinvasive bacteria into the intestinal epithelial cell is the key to a successful invasive process. The ability of *Y. enterocolitica* to invade epithelial cells is an important correlation of pathogenicity [8]. The invasive process includes a major signalling process that an invasive microorganism may provoke to force its way into a nonphagocytic cell, and then disrupting and invading the intestinal barrier, a process that involves interaction with other cellular components of this barrier. There are essentially two major mechanisms of bacterial epithelial cell internalization [74] The "zippering" process corresponds to tight enclosing of the bacterial cell by the mammalian cell membrane, involving a surface bound bacterial protein binding an adherence molecule of the mammalian cell surface with high affinity—that is, the invasin (Inv) of *Yersinia* binding integrins of the β1 family of mammalian cell surface [75]. One reason that strains of biotype 1A have been considered to be avirulent is that they invade tissue culture cells to a lesser extent than pYV-bearing strains [69, 76]. However, paradoxically, some pYV-bearing strains themselves may retard mammalian epithelial cell invasion via the effects of translocated Yops on cytoskeletal proteins [42] as well as some biotype 1A strains are positive for the *ail* gene encoded an outer membrane protein that may contribute to epithelial cell adhesion and invasion [77] (Figure 3).

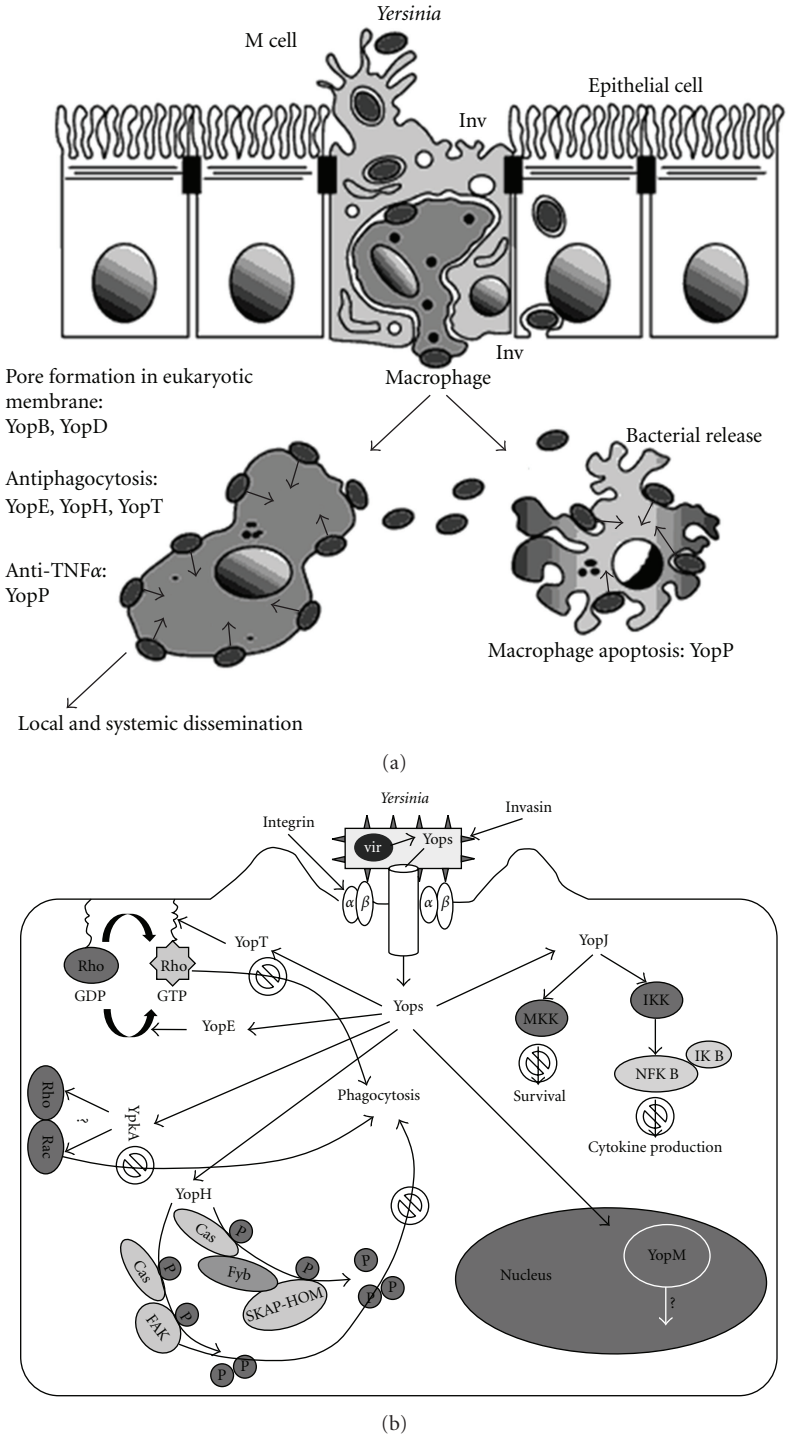

Figure 4: Physiopathological scheme of *Yersinia* infection. The Yops are delivered into the host cells via a type III secretion system. YopH, a tyrosine phosphatase, dephosphorylates Cas and FAK (protein tyrosine kinase) in epithelial cells, and Cas, Fyb, and SKAP-HOM in macrophages that are involved in the assembly of cytoskeletal complexes required for phagocytosis [78]; YopT modifies the Rho family GTPases by inducing redistribution of the RhoA GTPase [79]; YopE inactivates the Rho family of GTPases involved in phagocytosis [80]; YpkA binds to Rac and Rho (function unknown). These four Yops alter or disrupt the actin cytoskeleton and thereby block phagocytosis. YopJ impairs activation of MAPKKs and NF-B, which induces apoptosis and inhibits cytokine production. YopM is translocated into the nucleus (function unknown; adapted from [13]).

4.4. Local and Systemic Dissemination. Y. enterocolitica usually causes a diarrheal disease, and sometimes systemic diffusion. *Yersinia* virulent strains cross the intestinal epithelium primarily through the FAE (follicle associated epithelial cell), in the Peyer's patches of the ileum [81]. Invasin (Inv), a 103 kDa outer membrane protein of *Yersinia* binds β1 integrins that are also expressed apically on M cells. Inv negative mutants still adhere to and invade M cells, but at a much lower level than the wildtype strain and their colonization potential for Peyer's patches is considerably reduced [82]. Other *Yersinia* surface proteins such as Ail, PsaA, and YadA may account for residual invasion of *inv* mutants [83]. After invasion process, *Yersinia* defend the attack by resident macrophages by expressing an antiphagocytic strategy mediated by a plasmid encoded type III secretion, of three protein effectors, YopH, T, and E, that disrupt cytoskeletal assembly required for phagocytosis process [84, 85]. *Yersinia* strains therefore remain extracellular in infected Peyer's patches and mesenteric lymph nodes, and then disseminate to cause local and systemic infection (Figure 4).

5. Conclusion

Yersinia enterocolitica is most often transmitted by consumption of contaminated food (most commonly raw or undercooked pork), unpasteurized milk or inadequately pasteurized milk, untreated water, or by direct or indirect contact with animals. The virulence of *Y. enterocolitica* strains mostly depends on the presence of pYV plasmid. *Y. enterocolitica* pYV-positive strains contain plasmid-mediated virulence genes involved in developing infection especially in gastrointestinal tract with the help of traditional chromosomal genes whereas pYV-negative strains are mostly noninfectious except heat-stable enterotoxin-producing strain.

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Research Article

A Selective Chromogenic Plate, YECA, for the Detection of Pathogenic *Yersinia enterocolitica*: Specificity, Sensitivity, and Capacity to Detect Pathogenic *Y. enterocolitica* from Pig Tonsils

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A new selective chromogenic plate, YECA, was tested for its specificity, sensitivity, and accuracy to detect pathogenic *Y. enterocolitica* from pig tonsils. We tested a panel of 26 bacterial strains on YECA and compared it to PCA, CIN, and YeCM media. Detection of pathogenic *Y. enterocolitica* was carried out on 50 pig tonsils collected in one slaughter house. Enrichment was done in PSB and ITC broths. Streaking on YECA and CIN was done in direct, after 24H incubation of ITC, after 48H incubation of PSB and ITC. All the plates were incubated at 30°C during 24 hours. Presence of typical colonies on CIN and YECA was checked, and isolates were biotyped. Pathogenic *Y. enterocolitica* strains showed an important growth on YECA with small and red fuchsia colonies while biotype 1A exhibited very few violet colonies. Enrichment in ITC during 48H gave the best performance for detecting positive samples in pathogenic *Y. enterocolitica*, and YECA could detect directly pathogenic *Y. enterocolitica* strains (2, 3, and 4). Use of YECA in combination with ITC generates a time-saver by giving a positive test in 72H.

1. Introduction

Y. enterocolitica is a common cause of acute enteritis in temperate and cold countries worldwide, including France. The main symptoms of human yersiniosis are diarrhea, fever, and abdominal pain. Bacteria usually remain in the intestinal tract, but may also invade their host, causing abscesses in deep organs and septicemia in patients with underlying conditions [1].

In 2009, yersiniosis was, for the sixth consecutive year, the third most frequently reported human zoonosis in the Europe, with a total of 8,354 confirmed cases [2]. *Y. enterocolitica* was the most common *Yersinia* species reported in human cases in European countries, accounting for 93.8% of all confirmed cases of yersiniosis [3].

Pathogenic *Y. enterocolitica* strains belong to biotypes 1B, 2, 3, 4, and 5, whereas biotype 1A strains are nonpathogenic and widespread in the environment [4]. In France and most other countries worldwide, biotype 4 is the most prevalent biotype isolated from humans (69%), followed by biotype 2 (30%) and biotype 3 (1%) [1].

Human infections most frequently occur as sporadic cases or small family-centered outbreaks [1]. *Y. enterocolitica* is transmitted by the fecal-oral route, and its principal reservoirs are animals. Pigs are considered the principal reservoir for the types of *Y. enterocolitica* pathogenic to humans, although other animal species, such as cattle, sheep, poultry, fish, deer, small rodents, cats, and dogs, may also carry pathogenic biotypes [4–9]. Contaminated drinking water is also reported as source of biotype 1B *Yersinia* infection [10].

The incidence of yersiniosis due to pork consumption in humans was recently estimated at 2.8 cases per 100,000 inhabitants per year in Europe [11]. This bacterium is the second most frequent contaminant of pig products, after *Salmonella* (3.3) and ahead of *Campylobacter* (2.1). Pigs do not develop clinical signs, but they do carry *Y. enterocolitica* in their oral cavity, on tongues and tonsils, and in lymph nodes, and excrete this bacterium in their feces

[12, 13]. Bioserotype 4/O: 3 is the most prevalent pathogenic bioserotype isolated from pigs [14–20].

Detection of *Yersiniosis* is carried out by using ISO 10273-2003 method [21]. This method is recommended for both food and pig tonsil analyses [22] but involves time-consuming enrichment steps followed by plating on selective media [23]. This method involves enrichment in two broths, peptone sorbitol bile (PSB) broth, and irgasan-ticarcillin-potassium chlorate (ITC) broth, followed by a streaking on two plates, cefsulodin-irgasan-novobiocin (CIN) agar plateand *Salmonella-Shigella*-sodium deoxycholate-calcium chloride (SSDC) agar plate, respectively. Moreover, incubation of PSB broth can take up to five days. Recently, authors proposed modifications of the method in order to simplify the detection of *Y. enterocolitica*. Van Damme et al. (2010) [20] showed that the use of a two-day incubation period at 25°C, instead of five days, for the PSB broth resulted in a significantly higher recovery rate of *Yersinia*. Fondrevez et al. (2010) [24] demonstrated that streaking onto a CIN agar plate from ITC broth, recovered a larger number of positive samples than the ISO method. In addition, Weagant (2008) [25] has developed a chromogenic medium, *Yersinia enterocolitica* chromogenic medium (YeCM), for the specific detection of pathogenic *Y. enterocolitica*. However, difficulties were encountered to isolate pathogenic *Y. enterocolitica* colonies among the non-*Y. enterocolitica* colonies when using YeCM just after the enrichment step. It is the reason why a method involving streaking from ITC broth onto a CIN agar plate, followed by the streaking of typical *Y. enterocolitica* colonies onto the chromogenic medium, YeCM, was proposed by Fondrevez et al. (2010) [24]. This method allowed separation of *Y. enterocolitica* strains which carried pathogenic biotypes (red bull's-eye-like on YeCM) from the nonpathogenic biotype, 1A (blue-purple on YeCM) but an additional step of 24 hours is then needed.

Other alternative methods using PCR [8, 26] for detecting *Yersinia enterocolitica* from food or tonsil have been published. While PCR can be useful to quickly detect suspected positive samples, only culture method enable to recover isolates.

In this work, we tested a new selective chromogenic plate, YECA, for its specificity and sensitivity. We tested its accuracy to detect pathogenic *Y. enterocolitica* from pig tonsils as *Y. enterocolitica* becomes a preoccupation in Europe's pig production.

2. Materials and Methods

2.1. YECA: Yersinia Enterocolitica Agar—Selective Chromogenic Medium for Pathogenic Yersinia enterocolitica Screening. YECA developed by AES Chemunex (Combourg, France) is described as a chromogenic plate which permits to isolate specifically pathogenic *Yersinia enterocolitica;* the typical colonies are small and red fuchsia. This coloration is due to the presence of colour indicator revealed by sugar fermentation. The presence of desoxycholate improves the red fuchsia coloration of the pathogenic *Y. enterocolitica* colonies. The chromogenic substrate and tryptophan in the media allow the differentiation of pathogenic *Y. enterocolitica* strains from the nonpathogenic *Y. enterocolitica* strains (biotype 1A) and a majority of enterobacteria.

2.2. Specificity of YECA. The specificity of YECA was tested against 26 strains listed in Table 1. These strains were *Yersinia enterocolitica*, *Yersinia*-like, and non-*Yersinia*. The following strains, *Morganella morganii, Pseudomonas sp.*, and *Serratia liquefaciens,* were obtained from nontypical colonies isolated from CIN after pig tonsil swab enrichment in ITC during the study of Fondrevez et al. (2010) [24]. Each strain was cultured in 5 mL of appropriated broth and incubation temperature for 24 hours.

The cultures were all adjusted to 4 McFarland, corresponding to a concentration of 10^8 to 10^9 cells per mL. Streaking was then performed (1) on CIN agar plate (*Yersinia* Selective Agar Base and *Yersinia* Selective Supplement, Oxoid, Basingstoke, UK), (2) on YeCM medium (prepared in the laboratory as described by Weagant [25] and, (3) on YECA (AES chemunex, Combourg, France).

We measure the specificity by screening if the expected results for the *Yersinia enterocolitica* strains were obtained, that is, small and smooth colonies, with a red centre and a translucent rim, on CIN, red bull's-eye-like colonies for pathogenic *Y. enterocolitica* and blue-purple colonies for the nonpathogenic *Y. enterocolitica* on YeCM, small (<1 mm) red fuchsia colonies for pathogenic *Y. enterocolitica* and small (<1 mm) violet colonies for the nonpathogenic *Y. enterocolitica* on YECA. Moreover, if growth of bacteria was observed on plate, we noted the importance of growth in a scale from 1 to 5; 1 was applied when we observed one to 5 colonies on the plate, 5 when colonies covered all the plate.

2.3. Sensitivity of YECA. *Yersinia enterocolitica* strains from biotype 1A (IP124), 2 (IP383), 3 (IP29228), and 4 (IP134) (purchased from Pasteur Institute, Paris, France) were incubated in 5 mL of Brain Heart Infusion (BHI, AES Chemunex, Combourg, France) broth during 24 h at 30°C. The overnight cultures were all adjusted to 4 Mc Farland corresponding to a concentration of 10^8 to 10^9 cells per mL. For each biotype, a tenfold dilution was then done in tryptone salt. Then 100 μL of the -5 to -10 dilutions were spread on PCA, CIN and YeCM plates, and 100 μL of the -1 to -10 dilutions were spread on YECA plates. All the plates were incubated at 30°C for 24 hours and enumeration of the colonies was then performed.

3. Detection of Pathogenic *Yersinia enterocolitica* from Pig Tonsils

The assay was carried out on 50 pig tonsils collected from a slaughterhouse in five times (10 tonsils per visit), and culture method used has been presented in Figure 1. From each tonsil, 10 g were cut in small pieces and put into a bag containing 90 mL of PSB broth (prepared in the laboratory, as described in the ISO 10273:2003 method). After stomaching, 10 μL were streaked directly onto YECA and CIN plates, and 1 mL was transferred in 9 mL of ITC broth. PSB and ITC were incubated at 25°C for 48 hours,

Table 1: Growth and color of colonies of strains used to test the specificity of YECA media.

Strains obtained from	Name of the strains	Growth* and color of colonies on CIN plate	Growth and color of colonies on YeCM plate	Growth and color of colonies on YECA plate
Yersinia RNC from Pasteur Institute (Paris, France)	*Yersinia enterocolitica* biotype 2 (IP383)	+++++ red with a translucent rim	+++++ red bull's-eye-like	+++++ small red fuchsia
	Yersinia enterocolitica biotype 3 (IP29228)	+++++ red with a translucent rim	+++++ red bull's-eye-like	+++++ small red fuchsia
	Yersinia enterocolitica biotype 4 (IP134)	+++++ red with a translucent rim	+++++ red bull's-eye-like	+++++ small red fuchsia
	Yersinia enterocolitica biotype 1A (IP124)	+++++ red with a translucent rim	+++++ blue-purple	+ violet colonies (5)
Collection of the Pasteur Institute (Paris, France)	*Yersinia aldovae* (CIP103162)	+++++ red with translucent rim	+++++ yellow/redwith translucent rim	+ small red fuchsia (1)
	Yersinia bercovieri (CIP103323)	+++++ red with translucent rim	+++++ yellow/redwith translucent rim	++ yellow/small red fuchsia
	Yersinia frederiksenii (CIP80.29)	+++++ red with translucent rim	+++++ blue to green	++ green/small red fuchsia
	Yersinia kristensenii (CIP80.30)	+++++ red with translucent rim	++++redwith translucent rim	++ pink//small red fuchsia
	Yersinia massiliensis (CIP109351)	+++++ red with translucent rim	+++++ green	++ green/small red fuchsia
	Yersinia mollaretii (CIP103324)	+++++ red with translucent rim	+++++ yellow/redwith translucent rim	+ small red fuchsia (1)
	Yersinia rohdei (CIP103163)	+++++ red with translucent rim	+++++ yellow/redwith translucent rim	+ pink (1)
	Yersinia ruckeri (CIP82.80)	No growth	No growth	No growth
Collection of the Pasteur Institute (Paris, France)	*Salmonella* Typhimurium (CIP55.43)	No growth	No growth	No growth
	Campylobacter jejuni (CIP70.2)	No growth	No growth	No growth
	Enterococcus faecalis (CIP55/42)	No growth	No growth	No growth
	Lactobacillus plantarum (CIP103151)	No growth	No growth	No growth
	Pseudomonas fluorescens (CIP525)	+++++ yellow	+++++ yellow	+ pink
	Brochothrix thermosphacta (CIP103251)	No growth	No growth	No growth
Field strains from Anses collection	*Listeria monocytogene*s	No growth	No growth	No growth
	Escherichia coli	No growth	No growth	No growth
	Staphylococcus aureus	No growth	No growth	No growth
	Klebsiella sp.	No growth	No growth	No growth
	Proteus mirabilis	No growth	No growth	No growth
Strains from Fondrevez et al., (2010)	*Morganella morganii*	+++++ yellow	+++++ yellow	++ yellow/pink
	Pseudomonas sp.	+++++ yellow	+++++ yellow	+ pink
	Serratia liquefaciens	+++++ pink with translucent rim	+++++ green	++++ green/blue/pink

* Growth was measured from no growth (absence of colonies) to 5 +++++ (important culture with numerous colonies).

before a second streaking onto YECA, and CIN. In addition, after 24 hours of enrichment in ITC broth, an extra streaking on YECA and CIN was performed. All the plates were incubated at 30°C for 24 hours.

Presence of typical colonies on CIN (small and smooth with a red centre and translucent rim) and on YECA (small and red fuchsia) were checked. At least two typical colonies per plate were streaked on YeCM, and these plates were

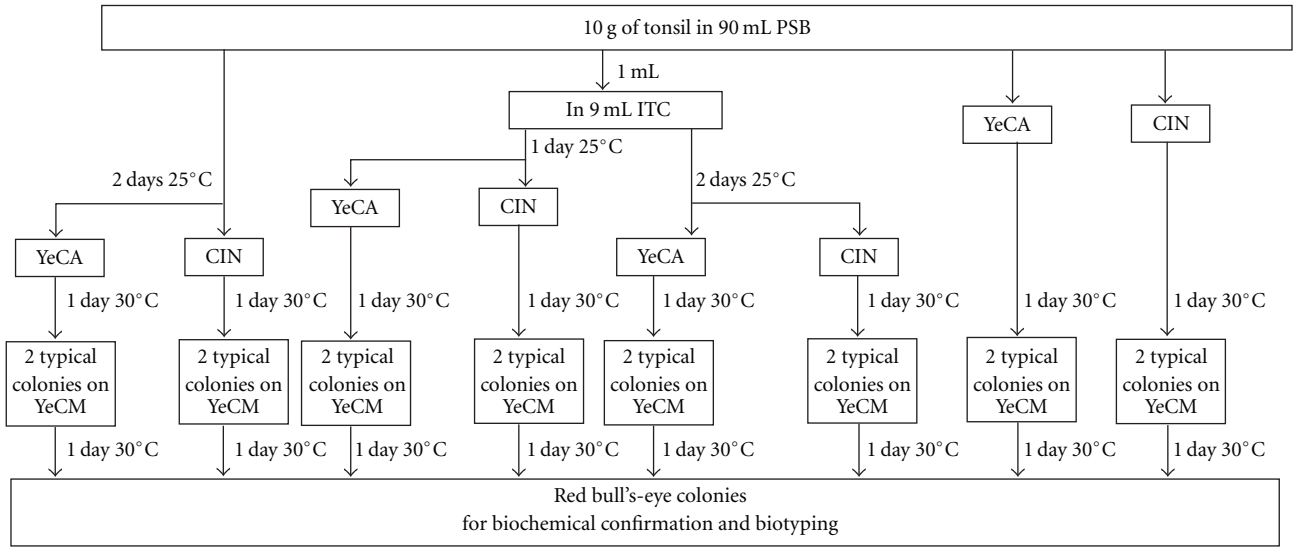

Figure 1: Overview of the methods used to isolate pathogenic *Yersinia enterocolitica* from pig tonsil in this study.

incubated at 30°C for 24 hours. This step on YeCM permitted to differentiate rapidly the pathogenic *Y. enterocolitica* (red bull's-eye-like colonies) from the nonpathogenic *Y. enterocolitica* (blue-purple colonies). Confirmation and biotyping was then done by biochemical assays as described in ISO 10273:2003 standard.

4. Results

4.1. Specificity of YECA (Table 1). The strains of *Yersinia enterocolitica* showed the expected characteristics on CIN and YeCM, that is, important growth for all the biotypes and, on CIN, small colonies, with a red centre and a translucent rim, and on YeCM, red bull's-eye-like colonies for biotype 2, 3, and 4 and blue-purple colonies for the nonpathogenic biotype 1A.

On YECA, the three pathogenic *Y. enterocolitica* showed an important growth with numerous small and red fuchsia colonies while the nonpathogenic biotype 1A had a very small growth on YECA. Only 5 violet colonies could be observed on YECA for this biotype while streaking was done from culture containing at least 10^8 cells per mL. YECA consequently exhibited a high inhibitor effect on the growth of the nonpathogenic biotype 1A.

On CIN, seven of the *Yersinia*-like strains grew as red colonies with translucent rim in fair number. Only *Yersinia ruckeri* was inhibited. Similar results were noted on YeCM with an inhibition of *Yersinia ruckeri* and a good growth of the other strains even though they grew as nontypical colonies.

Absence of growth was noted also for *Yersinia ruckeri* on YECA. The other *Yersinia*-likes strains were able to growth on YECA but the number of colonies was very small indicating that YECA had to high inhibitor effect on their growth. This inhibition is useful because we saw that the colony of *Yersinia aldovae* and the colony of *Yersinia mollaretii* had similar characteristics on YECA than *Yersinia enterocolitica*, that is, small red fuchsia. These two colonies were probably observed because streaking was carried out from a culture rich in cells, around 10^8 cells per mL.

For the 14 non-Yersinia strains, we observed for CIN, YeCM, and YECA an absence of growth or growth but as not characteristic colonies on these media.

These results showed that it is possible on YECA to differentiate the three pathogenic *Y. enterocolitica* from the panel of strains tested in this work.

4.2. Sensitivity of YECA (Table 2). The sensitivity of YECA against the four biotypes of *Yersinia enterocolitica* was compared to the one obtained on PCA, CIN, and YeCM using a 10-fold serial dilution of the four strains.

The sensitivity of YECA was identical to those of PCA, CIN, and YeCM for the pathogenic biotypes; enumeration was possible until the dilution −8.

But for the biotype 1A, colonies on YECA could be numerated only at the dilutions −1, −2, −3 while on PCA, CIN and YeCM, it was possible to count the colonies of this biotype until the dilution −8.

These results showed that YECA had the same sensitivity than selective and nonselective media. YECA allowed the detection of *Y. enterocolitica* strains carrying pathogenic biotype, specifically.

4.3. Detection of Pathogenic Yersinia enterocolitica from Pig Tonsils. Out of the 50 tonsils, pathogenic *Y. enterocolitica* were detected on CIN and YECA, respectively, from 17 and 15 tonsils after direct streaking, from 21 and 22 tonsils after ITC-24 hours, from 28 and 28 tonsils after ITC-48 hours, and from 8 and 5 tonsils after PSB-48 hours.

This work showed first that enrichment in ITC during 48 hours resulted in a significantly higher recovery rate of samples positive in pathogenic *Y. enterocolitica* compared to direct streaking, streaking after ITC-24 hours and streaking after PSB-48 hours. Secondly, the concordance between the

Table 2: Sensibility of YECA compared to PCA, CIN and YeCM against the four biotypes of *Yersinia enterocolitica*.

Dilution of the culture	Biotype 1A (IP124)				Biotype 2 (IP383)				Biotype 3 (IP29228)				Biotype 4 (IP134)			
	PCA	CIN	YeCM	YECA	PCA	CIN	YeCM	YECA	PCA	CIN	YeCM	YECA	PCA	CIN	YeCM	YECA
−1				150 VC				NN				NN				NN
−2				32 VC				NN				NN				NN
−3				2 VC				NN				NN				NN
−4				0				NN				NN				NN
−5	>200	>200	>150	0	>300	>300	>300	>300 RF	>300	>250	>250	>400 RF	>300	>300	>300	>300 RF
−6	53	39	46	0	34	53	50	49 RF	86	101	82	91 RF	70	77	78	63 RF
−7	3	3	2	0	3	5	6	6 RF	12	11	9	12 RF	5	7	8	10 RF
−8	1	1	1	0	0	1	1	1 RF	0	0	1	2 RF	0	2	0	1 RF
−9	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
−10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

VC: violet colonies; RF: red fuchsia colonies; NN: nonnumerable.

results obtained from CIN and YECA is high; the same number of positive tonsils was recovered after ITC-48 hours.

A total of 141 strains were collected on YECA and biotyped, 12 after PSB enrichment and 129 after ITC enrichment. Among the 141 strains, 135 were identified as biotype 4 (12 from PSB and 123 from ITC), two as biotype 3 and four as biotype 2. This result shows that YECA is able to detect these 3 pathogenic biotypes from naturally contaminated pig tonsils.

5. Discussion

At this day, the ISO 10273-2003 standard [21] is the reference method for isolating *Yersinia enterocolitica* from foods. This method is also recommended for pig tonsils analysis [22]. However, it involves time-consuming enrichment steps followed by plating on selective media [23]. This method involves enrichment in two broths, PSB and ITC, followed by a streaking on two plates, CIN and SSDC plates, respectively.

Cold enrichment in PSB broth was largely used for the clinical, food, and environmental samples. The major disadvantage of cold enrichment is the long period of incubation which is not appropriate for food analysis. Doyle and Hugdahl (1983) [27] showed that incubation in PSB solution during 1 to 3 days at 25°C was as effective as enrichment at 4°C during several weeks. This was recently confirmed by Van Damme et al. (2010) [20] who showed that the use of a two-day incubation period at 25°C, instead of five days, for the PSB broth, resulted in a significantly higher recovery rate of *Yersinia*.

Wauters et al. (1988) [28] developed an enrichment broth (ITC), derived from modified Rappaport, supplemented in Irgasan, Ticarcillin and potassium chlorate. The same authors indicated that enrichment in PSB broth gave better results for nonpathogenic strains, whereas enrichment in ITC broth gave better results for pathogenic strains. However, this broth proved to be effective bioserotype 4/O: 3 strains but inhibits bioserotype 2/O: 5, 27 strains [29, 30]. De Zutter et al. (1994) [31] modified ITC formula as to have a better recovery for bioserotype 2/O: 9 strains by decreasing the concentration of chloride potassium and in malachite green.

As indicated in the ISO 10273:2003 standard, *Yersinia enterocolitica* colonies on CIN agar are typically small and smooth, with a red centre and a translucent rim and, when examined with obliquely transmitted light, they are noniridescent and finely granular. On SSDC agar, *Yersinia enterocolitica* colonies are typically small and grey, with an indistinct rim, and are noniridescent and very finely granular when examined under obliquely transmitted light.

The SSDC agar is a modified SS agar with Sodium desoxycholate and calcium chloride in order to increase its selectivity [28]. *Yersinia* tolerates strong concentrations of this salt [32]. Moreover, the calcium chloride enhances the selection of the pathogenic strains of *Y. enterocolitica*, calcium dependent, in particular, bioserotype 4/O: 3 strains [28]. This agar is largely used because of its great selectivity and of its commercial availability. However, this medium does not always allow differentiating *Yersinia* from interfering flora such as *Morganella*, *Proteus*, *Serratia,* and *Aeromonas*.

It is Schiemann (1979) [33] who developed the medium CIN (Cefsulodin-Irgasan-Novobiocin) for the detection of *Y. enterocolitica*. The medium CIN is highly selective, especially against *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae,* and *Proteus mirabilis*. Colony morphology coupled with mannitol fermentation permitted discrimination of *Y. enterocolitica* from most of Gram-negative bacteria that can grow on this medium. Several comparative studies showed that CIN agar was the most selective medium for *Yersinia* spp. [34–36]. Micro-organisms able to ferment mannitol, like *Yersinia*, produce on CIN typical colonies after 24 hours (small and smooth colonies, with a red centre and a translucent rim). But *Citrobacter freundii, Enterobacter agglomerans,* and the species of *Aeromonas,* and *Klebsiella* produce colonies of similar morphology [37, 38]. However, users recognized that detection on CIN agar is easier since *Y. enterocolitica* has relatively more characteristic colony morphology on this medium (typical "bull's eye" appearance) compared to SSDC [20, 24]. However, Fondrevez et al. (2010)

[24] recommend the use of CIN after the enrichment in ITC broth. Tested on 900 pig tonsil swabs, the authors showed that this way recovered a larger number of positive samples than with the ISO 10272:2003 procedure: 14.0% of tonsils tested positive with the new method, versus only 9.1% with the modified ISO method.

These media, CIN and SSDC, moreover lack the ability to differentiate potentially virulent *Y. enterocolitica* from the nonpathogenic strains and other *Yersinia*. Only panel of biochemical tests (esculin hydrolysis, indole production, and fermentation of xylose and trehalose) as described in the ISO 10273:2003 method permits to identify the biotype.

Recently, Weagant (2008) [25] has developed a chromogenic medium (YeCM) for the specific detection of pathogenic *Y. enterocolitica*. This agar contains cellobiose as the fermentable sugar, a chromogenic substrate, and selective inhibitors for suppression of colony formation of competing flora. On this medium, pathogenic *Y. enterocolitica* strains grow as red bull's-eye-like colonies while nonpathogenic *Y. enterocolitica* grows as blue-purple colonies.

Direct use of this chromogenic agar after enrichment broth step was difficult because many nontypical colonies interfere with the visualization of the typical colonies. It is why Fondrevez et al. (2010) [24] proposed its use after the CIN step to quickly discriminate the nonpathogenic biotype from the pathogenic biotypes. While one more day is added in the detection, this method is less time consuming than the ISO 10273:2003 procedure and, with the use of YeCM, decreases the need for biochemical tests for confirmation and biotyping.

The European regulation concerning the zoonosis of food origin lies on the directive 2003/99/ that considers doing a monitoring of the principal agents responsible for food origin zoonosis, including *Yersinia enterocolitica*. In the last years, many countries showed an increasing interest in *Y. enterocolitica* epidemiology in pig production [17–19, 39, 40]. The various studies on this bacterium show also a real interest to propose other methods to detect it. Other alternative methods using PCR [8, 26] for detecting *Yersinia enterocolitica* from food or tonsil have been published. But while PCR can be useful to quickly detect suspected positive samples, only culture method enable to recover isolates which is necessary to study the spread of the bacteria from farms to humans. Recently, EFSA and the Members of the group of work *Yersinia enterocolitica* (2009) [41] proposed a national plan for monitoring *Yersinia enterocolitica* in pigs. It became necessary to have a simplified detection method which also could target directly the pathogenic biotypes responsible for human yersiniosis.

In this paper, we tested a new selective chromogenic plate, YECA, for its specificity, and sensitivity, and we tested its capacity to detect pathogenic *Y. enterocolitica* from pig tonsils.

YECA in this study showed a real capacity to favor the growth of the pathogenic *Y. enterocolitica* (Biotype 2, 3, and 4) with typical colonies, small, and red fuchsia. Growth of biotype 1A was much reduced with violet colonies. Absence of growth or light growth of nontypical colonies was observed for the *Yersinia*-like strains and non-*Yersinia* strains tested in this work. Moreover, numeration of pure culture of *Y. enterocolitica* strains on YECA was similar to those carried out on PCA, CIN and YeCM, except for biotype 1A for which high inhibition was observed. We observed that YECA exhibits a stronger inhibitor effect on the growth of the *Yersinia*-like strains while numerous colonies were observed on the chromogenic media YeCM developed by Weagant (2008) [25]. This is interesting because absence of other interferent bacterial flora on the media allows rapid visualization of the presence or absence of pathogenic *Y. enterocolitica* on YECA.

When tested from naturally contaminated pig tonsils, we observed a best performance for detecting positive samples after enrichment in ITC than in PSB and we obtained similar percentage of positive samples between CIN and YECA after enrichment in ITC during 48 hours. This result is consistent with the findings of Wauters et al. (1988) [28], indicating that enrichment in PSB broth gave better results for nonpathogenic strains, whereas enrichment in ITC broth gave better results for pathogenic strains. This result is also consistent with the work of Fondrevez et al. (2010) [24], showing that use of CIN after ITC recovered a larger number of positive samples than the use of CIN after PSB and the use of SSDC after ITC.

In this paper, isolates were confirmed as *Yersinia* and biotyped by biochemical assays as described in ISO 10273:2003 standard. This step was necessary to separate pathogenic strains from nonpathogenic strains. CIN does not differentiate biotype 1A from the pathogenic biotypes while YECA could detect directly pathogenic *Y. enterocolitica* strains. This indicates that use of YECA decreases the need for biochemical tests for confirmation and biotyping.

From naturally contaminated pig tonsils, it could be possible to isolate the three pathogenic biotypes 2, 3, and 4 on YECA after ITC enrichment; biotype 4 representing 95.7% of all isolates. In the study of Fondrevez et al. (2010) [24], the most prevalent biotype was also biotype 4 (80.2% of all isolates), followed by biotype 3 (19.4% of all isolates). But no biotype 2 strains were detected in its study probably because ITC broth and CIN plates both favour the growth of biotype 4 [31, 42]. The results of our study seem to put forward that YECA could have a better capacity for detecting biotype 2 strains than CIN but this has to be confirmed on a higher number of samples.

In three days, it was possible to detect pathogenic *Y. enterocolitica* strains from pig tonsils when using YECA after ITC. Consequently, combination of ITC enrichment and YECA detection generates a timesaver by giving a positive test for pathogenic *Yersinia enterocolitica* in 72 hours.

In conclusion, we have described a simplified method that efficiently detects pathogenic *Y. enterocolitica* in pig tonsils and that it is less time consuming than the ISO 10273:2003 standard.

In this study, we used this method on pig tonsils as *Yersinia enterocolitica* becomes a preoccupation in Europe's pig production, but studies has to be carried out for testing it on foods from animal or vegetal origin. Moreover, the chromogenic media could be tested on human faecal samples to detect human yersiniosis.

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