Molecular Characterization and Antibiotic Resistance of Yersinia Entrocolitica Strains Isolated from Fish

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Abstract. *Yersinia enterocolitica*, a widespread food and water-borne pathogen, is responsible for diseases in humans and animals. The aims of this study were to determine the molecular epidemiology of *Y. enterocolitica* strains isolated from, as well as, to investigate their pathogenic potential and assess the antibiotic resistance. Twelve *Y. enterocolitica* strains isolated from diseased fish were studied. Genotypic diversity was analyzed by ERIC-PCR and 16S rDNA-RFLP, virulence genes were assessed by PCR method. Kirby-Bauer disk diffusion method was applied for the antibiotic resistance profile. The results indicated that (i) The 12 strains were grouped into five clusters by ERIC-PCR with similarity ranging from 81.8% to 100%, while four RFLP types were identified by 16S rDNA-RFLP with similarity ranging from 83.3% to 100%. (ii) Genes *ail* and *intB* were detected in all strains, whereas, *virF* and *ystB* were found at high probability of 83.3%, 75%, respectively, *yadA* was 41.6%. (iii) The isolated strains showed antibiotics resistance as follows, lincomycin (100%), sulfafurazole (41.6%), cephalothin V (66.7%), rifampicin (75.0%) compound sulfamethoxazole (8.3%), streptomycin (8.3%) and rocephin (8.3%), but all were sensitive to gentamicin, kanamycin, tetracycline and enrofloxacin. It will benefit the further study of pathogenesis and prevention of *Y. enterocolitica* in.

1. Introduction

Yersinia entrocolitica is a widespread zoonotic pathogen that connected to yersiniosis disease in humans and animals. It is responsible for the intestinal diseases including acute terminal ileitis, enterocolitis with an inflammatory diarrhea and the extra-intestinal manifestations such as reactive

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arthritis, erythema nodosum, infected mycotic aneurysm, axillary abscessesbux, respiratory ract infection, urinary tract, and endocarditis [1-2].

Epidemiology studies of *Y. entrocolitica* strains previously have largely depended on biochemical, serotyping, antibiotic susceptibility and phage typing. However, these techniques are limited by their low reproducibility and discrimination power. Therefore, alternative methods have been attempted, most of them are DNA-based molecular techniques. They give the information on the source of infection, cross-transmission, as well as the geographical and host distributions. Random amplified polymorphic DNA(RAPD), analysis restriction enzyme analysis of plasmids/chromosomes (REAP/REAC), ribotyping were used to characterize the *Y. enterocolitica* strains [3-4]. In previous studies, pulsed-field gel electrophoresis(PFGE), enterobacterial repetitive intergenic consensus-PCR(ERIC-PCR) and repetitive intergenic palindromic sequence(REP -PCR) have been reported for typing the *Y. enterocolitica* isolates [5-7].

The pathogenicity of *Y. enterocolitica* strains has been attributed to various genes presented in a highly conserved 70-kb virulence plasmid (pYV/pCD) and the chromosome, as well as the high pathogenicity island (HPI). The pYV plasmid virulence genes, such as *yadA*, *virF*, *ysa* and *tccC*, contribute to the bacteria survival and proliferate in host cell [8]. Chromosomally borne genes *ail* and *inv* allow *Y. enterocolitica* to adhesive, invade and translocate across the intestinal epithelium, while the *yst* gene encoding the Yersinia stable toxins might be contributed to diarrhea associated with yersiniosis. Furthermore, high-path ogenicity island (HPI) genes such as *ybt*, *irp*, *intB* play a significant role in iron acquisition in pathogenic strains.

In China, some virulence genes have been reported in *Y. enterocolitica* strains isolated from diarrhea patients, animals, foods and natural environment [9-11]. Epidemiology of *Y. enterocolitica* strains was mainly performed with pulsed-field gel electrophoresis to investigate the molecular subtypes, which presented a high discrimination power, but expensive and time-consuming [12]. Nevertheless, there was few data about *Y. enterocolitica* strains isolated from fish. In this study, we analyzed the strains of *Y. enterocolitica* isolated from various types of fish in Sichuan province, inspect the genotype diversity, virulence genes and antibiotic resistance of *Y. enterocolitica* strains. It will benefit the further understanding of epidemiology of *Y. enterocolitica* strains.

2. Materials and methods

2.1. Bacteria strains

Twelve strains of *Y. enterocolitica*, isolated from fish with typical symptoms, were analyzed in this study. The isolates were directly cultured on Cefsulodin-Irgasan-Novobiocin Agar-1 (CIN) supplemented with ampicillin, chloramphenicol and diphenyl or on Modified Y medium, the inoculated media were incubated at 28°C for 18~24 h. These strains were confirmed as *Y. enterocolitica* by morphological appearance and biochemical tests [13], and 16S rDNA gene sequencing [14-15]. All the diphenyl identified strains were belonging to serotype O:8 which were frozen at -80°C with 20% glycerol and conserved in CIN-1 Agar (plates) at 4 °C for further study. All experiments involving live fish were approved by the Animal Ethics Committee at Southwest University for Nationalities.

2.2. Extraction of genomic DNA

Genomic DNA was extracted by using the TIANamp Bacteria DNA Kit (QIAGEN). The purity and concentration of DNA preparations were estimated spectrophotometrically at 260 and 280 nm.

2.3. 16S rDNA-RFLP typing

The 16S rDNA gene sequences were amplified from isolated strains using universal primer: Reverse primer (5'-AGAGTTTGATCATGGTCAG-3') and Forward primer

(5'-ACGGCTACCTTGTTACGACTT-3'). The PCR reaction system contained 10 pmM primers, 1 ×QIAGEN PCR mixture (QIAGEN) and 50 ng genomic DNA. The amplification program were described by Charbonneau et al.(2012). The PCR products were digested with *Taq I* (TaKaRa) for 16S rDNA-RFLP typing

2.4. ERIC-PCR typing

The PCR reaction system contained 10 pmM primer, 1.0 μL Taq DNA polymerase (TaKaRa), 2.5 μL 10×PCR Buffer (Mg^{2+} Free), 2.5 mM $MgCl_2$, 1.0 μL dNTP Mixture and 50ng genomic DNA. The primers used were ERIC-Forward (5'-AAGTAGTGACTGGGGTGAGCG-3') and ERIC-Reverse (5'-ATGTAAGCTCCTGGGGATTCAC-3')[16]. All PCR reactions were repeated twice for each *Y. enterocolitica* strain.

2.5. Computer data analysis

The gel photographs of ERIC-PCR and 16S rDNA-RFLP were analyzed by Gel-Pro analyzer 4.0. Dendrograms were constructed by the unweighted pair group method (UPGMA) using NTSYS-pc software (version 2.10e).

2.6. PCR amplification of virulence genes

The PCR reaction contained 10 pmM primers, 1×QIAGEN PCR mixture (QIAGEN) and 50 ng genomic DNA. PCR reactions were 94 °C for 4 min, followed by 35 cycles of 94 °C for 45 s, graded temperature (45 °C to 65 °C) for 45 s, and 72 °C for 40 s, and incubated at 72 °C for 5 min.The primer sequences, size of products, annealing temperature and the references sequences were summarized in Table 1.

Table 1. Primers of the virulence genes.

Genes	Primers	Primers sequence (5'-3')	Amplicon Size (bp)	GenBank accession No.	Annealing
ail	Forward Reverse	taa tgt gta cgc tgc gag gac gtc tta ctt gca ctg	351	M29945	50
ystB	Forward Reverse	gta cat tag gcc aag aga cg gca aca tac ctc aca aca cc	200	GU229276	60.3
virF	Forward Reverse	ggc aga aca gca gtc aga cata ggt gag cat aga gaa tac gtc g	561	NC004564.	45
yadA	Forward Reverse	ctt cag ata ctg gtg tcg ctg t atg cct gac tag agc gat atc c	800	NC004564	58.5
intB	Forward Reverse	tgc gcc atg cgg tcc atc ggt gca taa gat tct cgg	714	NC008800	50

2.7. Antibiotics resistance of strains

Drug resistance of *Y. enterocolitica* strains were evaluated by Kirby-Bauer's disk diffusion method [17]. The tested antimicrobial drugs and concentration were as follows, kanamycin(30 μg), gentamicin(10 μg), streptomycin (10 μg), tetracycline (30 μg), enrofloxacin (5 μg), rocephin (30 μg), cephalothin V (30 μg), polymyxin (300 μg), lincomycin (2 μg), rifampicin (5 μg), compound sulfamethoxazole (75 μg), sulfafurazole (300 μg).

3. Results

3.1. 16S rDNA-RFLP analysis

The digestion of 16S rDNA PCR products by *Taq I* yielded different fragments sizes from 100 to 700 bp and a common band of 350 bp appeared in all strains (Figure 1). Dendrogram showed that four groups were clustered for 12 *Y. enterocolitic* isolates (Figure 2). The first group included *Y. enterocolitic* strains YE01, YE04, YE07, YE08, YE09, YE11, YE12, and the similarities were 100% among them. The second group included YE06 and YE10, with similarity of 100%. The third group contained YE02 and YE03 with similarity of 100%. But the isolate YE05 comprised the last group only shared 83.3% similarity to other groups. Based on these results, it inferred that strains of the same serotype showing different PCR-RFLP profiles.

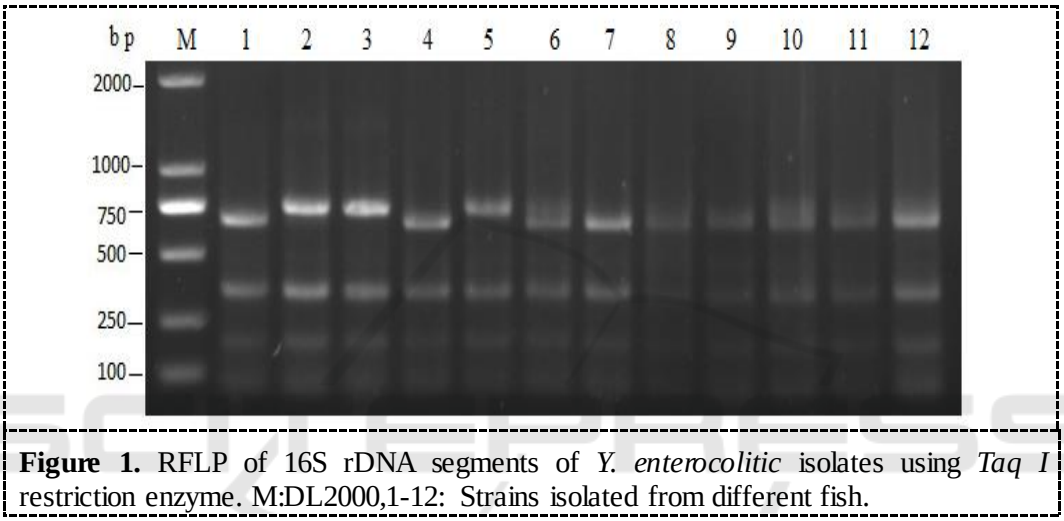

Figure 1. RFLP of 16S rDNA segments of *Y. enterocolitic* isolates using *Taq I* restriction enzyme. M:DL2000,1-12: Strains isolated from different fish.

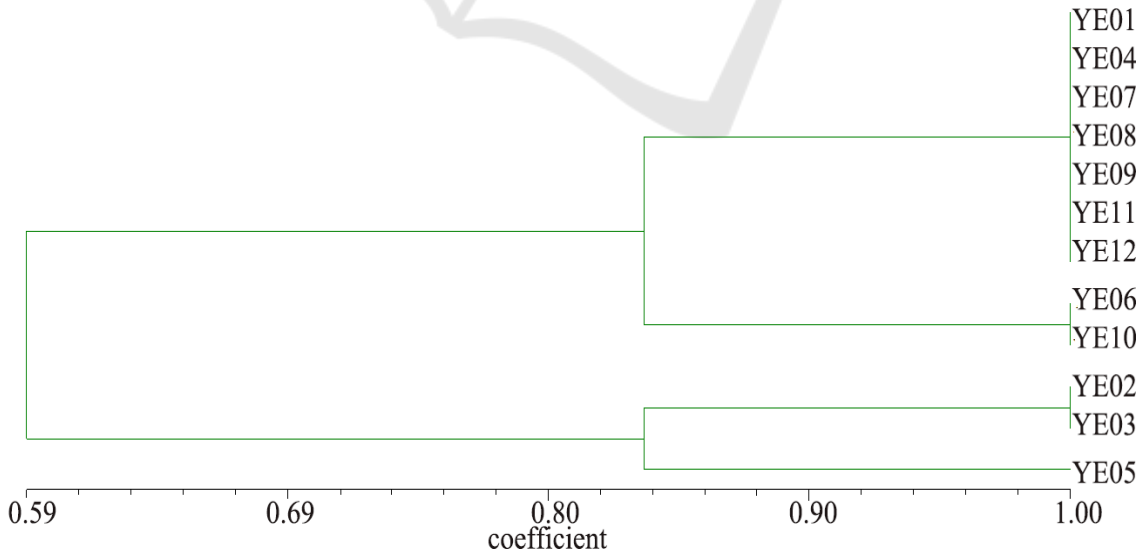

Figure 2. Dendrogram based on RFLP of 16S rDNA segments of *Y. enterocolitic* isolates using *Taq I* restriction enzyme.

3.2. ERIC-PCR analysis

DNA fragments generated by ERIC-PCR were composed of 6 to 10 amplification bands ranging from 200 to 2,500 bp (Figure 3). All isolated strains were differentiated into five electrophoretic patterns (Figure 4). The vast majority of isolates were divided into cluster I with 33.3% of the 12 strains, Strains YE06, YE07, YE08 and YE09 in cluster I had exhibited 100% similarity, and YE01 (90% similarity). Cluster II was comprised by YE12 showed a different banding pattern to the other isolates. YE03 and YE04 in cluster III isolated from *Yellow catfish* in the same farm were grouped together with 100% similarity. In cluster IV, YE10 and YE11 isolated from different host in Xindu aquafarm presented similar banding patterns. Particularly, YE02 and YE05 collected from *Carassius auratus* and *Yellow catfish* in different aquafarms exhibited 90% similarity, which were clustered to cluster V.

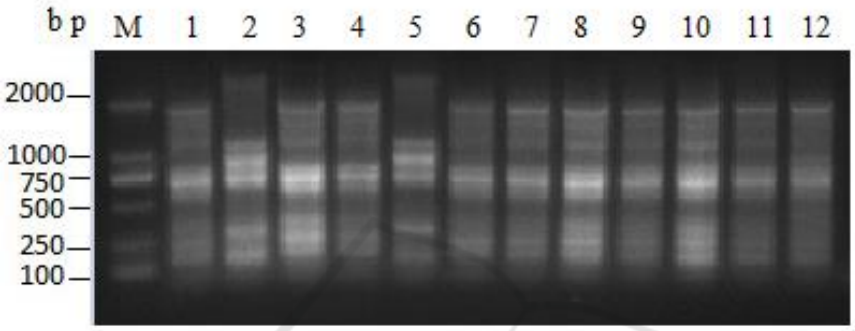

Figure 3. Amplification of ERIC genes for twelve *Y. enterocolitica* strains M:DL2000,1-12: Strains isolated from different fish.

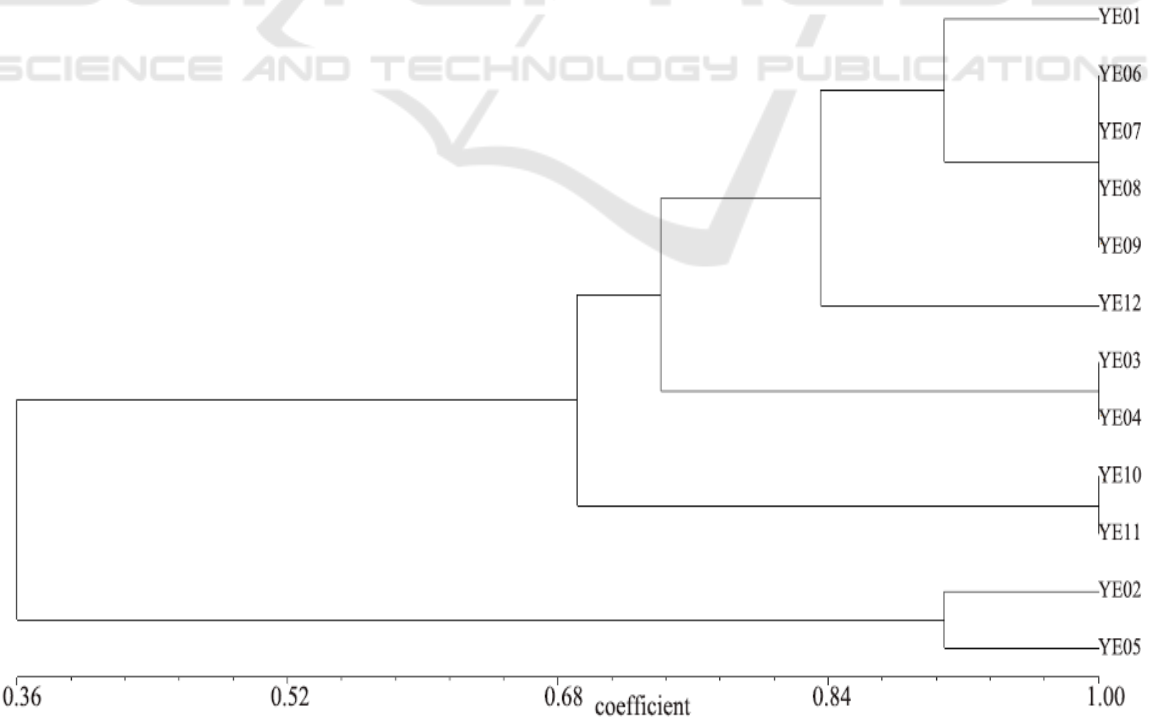

Figure 4. Dendrogram produced by UPGMA based on Jaccard coefficient representing genetic relationships between *Y. enterocolitica* isolates based on ERIC-PCR analysis.

3.3. Virulence genes

All isolated strains were screened for the virulence genes (Table 2). Genes *ail* and *intB* harbored in all strains (100%), while genes *virF*, *ystB* and *yadA* were respectively 83.3% (10/12 strains), 75% (9/12 strains) and 41.6% (5/12 strains). We also found that 83.3% of *Y. entrocolitica* strains presented at least two virulence genes. Interestingly, in this study, gene *ystB* was found correlated to gene *ail*, *virF* and *yadA*.

3.4. Antimicrobial resistance

Drug resistance of *Y. entrocolitica* isolates analyzed in this study showed that all strains were resistant to lincomycin. sulfafurazole (41.6%), cephalothin V (66.7%) and rifampicin (75.0%). While, a slightly antibiotic resistance was observed in compound sulfamethoxazole (8.3%), streptomycin (8.3%) and rocephin (8.3%). Intermediate susceptible to polymyxin B was distributed among all the isolated strains. No resistance was observed in gentamicin, kanamycin, tetracycline and enrofloxacin. More than 33.3% of the strains were resistant to two classes of antimicrobial agents in this study.

4. Discussion

It is well known that *Y. enterocolitica* is an emerging food-borne pathogen that is widespread throughout the world. This bacterium is acquired primarily through the consumption of contaminated food and water. Therefore, epidemiological investigations play an important role in elucidating contamination routes and establishing the implementation of control and prevention measures [8,18]. Elaborated the relationship of *Y. enterocolitica* strains isolated from animals, humans and environment, but no molecular typing study has been reported in aquaculture fish.

In this study, the relationship between twelve isolates was analyzed by ERIC-PCR and 16S rDNA-RFLP methods. Majority of isolates from different farms manifested different ERIC profiles, which indicated its potential usefulness in epidemiological studies for *Y. enterocolitica*, which was consistent with the study of Wojciech [7], who used ITS profiling, REP-PCR and ERIC-PCR to assess the genomic diversity among 35 *Y. enterocolitica* strains isolated from humans, pigs and foxes. ERIC-PCR was regard as an effective method to discriminate *Y. enterocolitica* strains in a study of 81 biovar 1A strains isolated from clinical and nonclinical trails and in another study of 81 strains isolated from India, Germany, France and USA[1-2]. Similarly, in the study carried out by Paixão[19], 61 strains of *Y. enterocolitica* isolated among pigs and slaughterhouses in Brazil were characterized by SE-AFLP, ERIC-PCR and PFGE techniques, in which ERIC-PCR appeared more useful for separating the isolates among the different serotypes, though with a slightly lower discriminatory power than the other techniques.

The universal primers of 16S rDNA/rRNA were conservative molecules, universally distributed and functionally constant. Thus, the PCR-restriction fragment length polymorphism analysis by 16S rDNA/rRNA technique had been used to discriminate the *Bradyrhizobium* and *Rhizobia* strains isolated from soybean and to assess the genetic diversity of *Aeromonas veronii*, *Thermophilic* bacterial and *Arcobacter spp.* [20-23]. In this study, most strains isolated from the same farms were grouped together. The results were almost consistent with ERIC, suggesting that these strains were not only genetically related, but also associated with their living environment and feeding conditions. Strains belonging to RFLP cluster did not identically belong to the same ERIC cluster, it might be caused by high sequence divergence of 16S rDNA genes among different strains. Additionally, it was interesting to note that all isolated strains were shared a common band of 350 bp in RFLP profiles. This region would be a favourable clue for the development of genetic amplification assay for identification and diagnostic purpose. Both ERIC-PCR and 16S rDNA-RFLP methods gave a high level of homogeneity, implying that these twleve *Y. enterocolitica* strains might have descended from a common ancestor, which indicated they were useful techniques for studying the prevalence of *Y.*

enterocolitica from diverse fishes. While, these techniques presented a distinct advantage with efficiency, simplicity, lower costs and universality of primers used [24].

Y. enterocolitica invasion factor encoding gene *ail* is presently stable in chromosomal that attributed to pathogenic biotypes Th reported ystB gene to be presented us, some researchers have established detect methods based on *ail* gene for *Y. enterocolitica* detection [25]. The result of this study indicated that all *Y. enterocolitica* strains tested were all positive for *ail* gene. The result was consistent with a previous study in which 172 *Y. enterocolitica* strains isolated from conventional and antimicrobial-free pig production systems from different geographic regions [26]. However, the *ystB* gene, producing an enterotoxin, presented in 75% (10 of 12) of the isolates which was much higher than the results reported by Tadesse et al., and in contrast to other studies, which reported that *ystB* gene was presented only in biotype 1A strains [27]. *Y. enterocolitica* strains in this study were isolated from aquatic fish, while other studies were from animals and humans, it might be related to the distribution of isolated species.

Plasmid-borne genes *yadA* and *virF* were selected as markers in this study. Interestingly, most of the isolated strains were positive for *virF* (83.3%), while only 5 of them positive for *yadA* (41.6%). This was accord *with* previous studies [28], in which *Y. enterocolitica* strain *virF* was positive while *yadA* was negative. It suggested that perhaps virulence genes *virF* and *yadA* were not located on the same plasmid every time. Moreover, the virulence plasmid could be easily lost because of repeatedly cultivation or storage in room temperature for a long time. On the other hand, plasmids acted as mobile elements and were transferable between bacterial strains which could be likely gained or lost under selective pressure. In addition, amount of virulence genes were clustered in a genomic island called Yersinia high-pathogenicity island (HPI) in *Y. enterocolitica* 1B isolates. Genes harbored on the island, including the mobility module locus termed asn-int, might play a role in encoding for biosynthesis and transportation of yersiniabactin (Ybt), and mediated iron acquisition [29]. Gene *intB*, a unidirectional site-specific recombinase, was the main part of the genetic dissemination machinery. In this study, all the strains were positive for *intB*, manifesting that this gene had been integrated stability in the bacteria strains. Therefore, the isolates might be potential pathogenic to fish, while further studies were needed for validating the pathogenesis in aquatic animals.

Antimicrobials are not usually recommended to treat uncomplicated yersiniosis, however, in more severe cases, such as focal extra-intestinal infection or septicaemia, use of antimicrobials is required [30]. In present study, the antimicrobial resistance of isolates was tested against some antibiotic drugs, which were common used in human and aquatic treatments. The antimicrobial resistance profile showed that 100% of the strains were resistant to lincomycin, which played a role as erythromycin. Likewise, Estrella et al. found a very high resistance rate (100%) of this agent among pork and chicken in Italy [21]. In previous studies, *Y. enterocolitica* strains were sensitive to polymyxin B [31]. However, in this study, all isolates were intermediately sensitive to polymyxin B. It insinuated that the percentage of *Y. enterocolitica* strains resistant to polymyxin B had been increased over times.

A few strains were resistant to streptomycin agreed with previously published results, but differed from the observation that the *Y. enterocolitica* organisms collected from pork and chicken in Italy, during 2006 and 2007 [32-33]. Additionally, a lower resistance level to trimethoprim/ sulfamethoxazole (8.3%) was observed, in contrast to a study in Latvia, which reported a high sensitivity of trimethoprim/sulfamethoxazole among *Y. enterocolitica* strains in slaughter pigs. The resistance against sulfamethoxazole (41.6%) in this study was much higher than previous studies observed by Bonke et al. [34]. and Meyer et al.[35]. Based on these data, it might be the use of antibiotic agents varied in different countries and fish-raised farms.

5. Conclusions

The genotype diversity, virulence genes and antibiotic resistance of *Y. ertrocolitica* strains isolated from fish were studied by molecular biology and pharmacological methods. Fish might become the

source of *Y. enterocolitica* infection. *Y.* enterocolitica may spread between fish and human, since fish were important food for human. Moreover, the antibiotic resistance of *Y. enterocolitica* strains was increasing. It was necessary to strengthen the monitoring of *Y. enterocolitica* infection in fish culture. Further studied would be focused on the pathogenicity and pathogenesis of *Y. enterocolitica* in fish.

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