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Heterogeneity of *Yersinia ruckeri* Isolated from Rainbow Trout (*Oncorhynchus mykiss*) Infected with Enteric Red Mouth Disease

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ABSTRACT

Regarding the increasing prevalence of antimicrobial resistance as a global menace, typing procedures are of great importance in epidemiological surveys. In the current study, interspecies differences of *Yersinia ruckeri* (*Y. ruckeri*) isolates were detected by antimicrobial susceptibility profiling and molecular traits to determine the relationship between isolates. The current study was conducted on 27 *Y. ruckeri* isolates collected from 40 infected rainbow trout (*Oncorhynchus mykiss*) with clinical enteric red mouth disease in Mashhad, Iran. Interspecies differences of all isolates were detected by antimicrobial susceptibility profiling using disk diffusion method, Repetitive Extragenic Palindromic PCR (REP-PCR), Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR), and Random Amplification of Polymorphic DNA PCR (RAPD-PCR) to determine the relationship between isolates. Simpson's diversity index was calculated for each typing technique. The most phenotype resistant was against ampicillin, chloramphenicol, and lincomycin. Furthermore, enrofloxacin and ciprofloxacin showed the highest activity (100%) against isolates. According to the results, the highest diversity index was observed in the REP-PCR method (D = 0.91). In conclusion, REP-PCR can be a powerful technique for epidemiological studies.

Keywords: Antimicrobial susceptibility, Enteric red mouth disease, Epidemiological study, Molecular typing, *Yersinia ruckeri*

INTRODUCTION

Enteric red mouth disease (ERM) is a highlighted infectious disease in the rainbow trout (*Oncorhynchus mykiss*) farming industry that causes economic problems in many countries (Ummey et al., 2021). Clinical signs include dark coloration, hemorrhages in the mouth, the presence of pale soft liver and kidney, muscle degradation, swollen abdomen, and gastroenteritis (Wrobel et al., 2020). The disease is caused by *Yersinia ruckeri (Y. ruckeri)*, a Gram-negative rod-shaped coliform (Ohtani et al., 2019). The *Y. ruckeri* was initially isolated from rainbow trout in Hagerman Valley, the United States (Zorriehzahra et al., 2017), and is currently found worldwide (Duman et al., 2017). Dissemination of ERM infection could be prevented using the powerful molecular typing method. Therefore, there is a need to determine a robust technique (Sedighi et al., 2020).

There are various methods for the differentiation and characterization of bacteria. These methods can be divided into phenotypic and molecular techniques, including Enterobacterial repetitive intragenic consensus (ERIC), random amplification of polymorphic DNA (RAPD), and repetitive extragenic palindromic (REP-PCR), which has higher discrimination power than phenotypic methods (Zorriehzahra et al., 2017). The REP-PCR depends on primers that hybridize to the short repetitive sequence elements dispersed throughout the bacterial genome to generate DNA fingerprints leading to strain discrimination (Priyambada et al., 2017).

The RAPD-PCR is based on randomly amplifying DNA segments (Duman et al., 2017). The ERIC elements are repetitive sequence elements in bacterial genomes (Otokunefor et al., 2020). REP-PCR primer is complementary to interspersed palindromic repetitive sequences. PCR amplifies different sizes of DNA fragments consisting of unique DNA sequences are between these palindromic repeats (Sharma et al., 2020). In the current study, interspecies differences of *Y. ruckeri* isolates from northeast Iran, Mashhad, were detected by antimicrobial susceptibility profiling and molecular traits to determine the relationship between isolates in this region. Moreover, Simpson's diversity index was calculated to identify this survey's most effective typing method.

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MATERIALS AND METHODS

Ethical approval

The authors declare that all practical procedures were done according to the Ethics Committee of the Faculty of Veterinary, Ferdowsi University of Mashhad, Mashhad, Iran.

Bacterial isolates and identification

From February to May 2020, 40 rainbow trout with an average weight of 498 g suffering from ERM and clinical symptoms of yersiniosis were transferred from three fish farms in Mashhad to the Microbiology Laboratory of Veterinary Faculty of Ferdowsi University of Mashhad. As Akhlaghi and Sharifi Yazdi (2008) explained earlier, the infected fish were dissected under aseptic conditions. Homogeneous suspensions of gills, liver, and kidney were streaked on Trypticase Soy Agar (TSA), and Nutrient agar with 5% blood (Columbia Blood Agar, HiMedia, India) plates with 48 hours incubation at 25 ± 2℃. The standard biochemical tests were performed for presumptive *Y. ruckeri* colonies. In this regard, the isolates were identified with gram-negative coccobacillus morphology and growth of lactose negative colonies on MacConkey agar (HiMedia, India) at 25 ±2℃, positive Catalase, negative Oxidase, positive Mobility, glucose utilization in Triple Sugar Iron agar (TSI), Indole produce, Urease positive test, positive methyl red (MR), negative Voges-Proskauer (VP), citrate use (Simon's citrate agar), Gelatin hydrolysis, fermentation of mannitol and glucose (Tkachenko et al., 2019). The confirmation of *Y. ruckeri* isolates was done by PCR using 16S rRNA primers. A total of 27 different *Y. ruckeri* isolates were isolated, Samples 1 to 8 were from Farm A, samples 9 to 17 were from Farm B, and samples 18 to 27 were from Farm C. All *Y. ruckeri* isolates after verification were kept in nutrient broth with 15% glycerol at -70℃ in the microbiology laboratory of the Veterinary Faculty, Ferdowsi University of Mashhad, Iran.

Antimicrobial susceptibility test

The Kirby-Bauer method was done to determine antimicrobial susceptibility as recommended by VET04 (CLSI, 2020). For this purpose, a few colonies from 24-hour culture were suspended by a sterile cotton swab into 5 ml saline to prepare 0.5 McFarland concentration (1.5 × 108 CFU/ml). Then, the swab was squeezed in a bottle and cultured on Mueller-Hinton agar (HiMedia, India), and antibiotic discs were placed on it. Ten antibiotic discs (Mast Group, UK), which interested in veterinary were tested compromise ampicillin (10 µg), gentamicin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), enrofloxacin (5µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), lincomycin (2 µg), linco-spectin (15.2 µg), oxytetracycline (30 µg) and cephalothin (30 µg). Results were interpreted according to the guidelines of CLSI (2020), and isolates were divided based on their susceptibility profile. The reference strain *Escherichia coli* ATCC 25922 was used as quality control.

Genetic characterization

Isolates were cultured on Nutrient agar (Merck, Germany) and incubated at 25 ± 2℃ for 24 hours. Bacterial DNA was extracted by boiling methods. Briefly, 10 bacterial colonies were taken from Nutrient agar and suspended in 500 µl of sterile distilled water. After that, the suspension was incubated in a boiling water bath for 15 minutes. Then, vials were kept at 4℃ for 10 minutes, centrifuged for 5 minutes at 14000 g, and the supernatant was used as the template for the PCR amplification (Ahmed and Dablool, 2017).

PCR information

Previously designed oligonucleotide primers were commercially synthesized by Cinnagen Corporation, Iran. Table 1 presents the properties of primers. PCR amplification was performed in 25 µl reaction containing 12.5 µl PCR 2X-Mastermix (containing Taq DNA polymerase 2 U, $MgCl_2$ 5 mM, dNTPs 0.4 mM, Cinnagen Co., Iran), 3 µl of DNA template (50 ng/reaction), 7.5 µl sterile distilled water, 400 nM (10 pM/µl) of each forward and reverse primer was combined.

Gel electrophoresis

The PCR products were run by electrophoresis in 1.8% agarose gel with 0.2 µl DNA Safe Stain (Cinnagen Co., Iran) and detected by Gel Doc (gel documentation system).

Data analysis

Determination, a powerful typing method that can discriminate studying isolates, is essential in epidemiological studies. The present study sought the power of discrimination for used techniques individually using a single numerical index of discrimination (D), based on Simpson's diversity index; accordingly, the following equation.

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

Where, N is the total number of isolates in the sample population, s refers to the total number of types, and n signifies the number of isolates belonging to the j^{th} type (Carriço et al., 2006).

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Table 1. The oligonucleotide primer's information was used for different PCR amplification.

Primer's name	Sequences (5′-3′)	Annealing (Tm)	Product size (bp)	References
Ruk1 Ruk2	CAG CGG AAA GTA GCT TG TGT TCA GTG CTA TTA ACA CTT AA	55°C	409	(LeJeune and Rurangirwa, 2000)
ERIC1 ERIC2	ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG-3	60°C	-	(Meacham et al., 2003)
REP-1 REP-2	IIIGCGCCGICATCAGGC ACGTCTTATCAGGCCTAC	45°C	-	(Martín-Lozano et al., 2002)
RAPD-1 RAPD-2	CCGCAGCCAA GAGACGCACA	36ºC	-	(Akhila et al., 2013)

RESULTS

Biochemical tests and confirmation PCR

All isolates indicated the same reaction to biochemical tests. These results revealed no significant differences between isolates. All 27 isolates were confirmed as a *Y. ruckeri* by amplifying 16S rRNA (Figure 1).

Antimicrobial susceptibility profiling

Table 2 illustrates the frequency of antimicrobial susceptibility. A total of 27 isolates were divided into six antibiotypes (Table 3), and the results were equal after three times repetition. The most phenotype resistance was obtained against ampicillin, chloramphenicol, and lincomycin. Simpson's diversity in this method was D = 0.75, equal to RAPD and lower than other methods used in the present study.

PCR analysis

Visual comparison of the REP-PCR banding results of 27 *Y. ruckeri* isolates showed ten reproducible fingerprint patterns. The multiple DNA fragments generated ranged in sizes between 150 and 2700 bps. RAPD technique produces five fingerprint patterns with bands ranging between 300 and 2300 bps. The ERIC method generated eight fingerprint patterns in which fragment sizes were between 150 and 1600 bps. The D values for this analysis were 0.72, 0.83, and 0.91 for RAPD, ERIC, and REP-PCR methods, respectively.

Analysis of dendrogram

GelJ software (version 2) was used to analyze the gel images and generate a dendrogram. The dendrogram was drawn based on a similarity matrix with the Dice method. According to the dendrogram with the discriminative power of 90% to assign the clusters, strains were classified into four clusters without any singleton in REP-PCR (Figure 2), three Clusters, and one singleton in RAPD-PCR (Figure 3), and three Clusters regarding ERIC-PCR (Figure 4), respectively.

Table 2. The antimicrobial susceptibility testing results of *Y. ruckeri* isolated from Rainbow trout Farms in Mashhad, Iran during 2020

	Antibiotics									
Interpretation	AMP	GEN	CHL	CIP	ENR	SXT	LIN	LSP	OXI	CEP
Susceptible	0	27	10	27	27	20	0	20	19	2
Intermediate	0	0	0	0	0	0	0	0	0	0
Resistant	27	0	17	0	0	7	27	7	8	25

AMP: Ampicillin, GEN: Gentamycin, CHL: Chloramphenicol, CIP: Ciprofloxacin, ENR: Enrofloxacin, SXT: Trimethoprim-sulfamethoxazole, LIN: Lincomycin, LSP: Linco-spectin, OXI: Oxytetracycline, CEP: Cephalothin.

Table 3. Profiling of antimicrobial resistance patterns of *Y. ruckeri* strains isolated from Rainbow trout Farms in Mashhad, Iran during 2020.

Isolation farms (Fisheries)	Antibiotic resistance patterns	Strain number
A	AMP, CHL, SXT, LIN, LSP, OXI, CEP	1, 3, 4, 5, 6, 7
A	AMP, CHL, SXT, LIN, LSP, OXI	2
A	APM, CHL, LIN, OXI	8
B	AMP, LIN, CEP	9, 11, 12, 13, 14, 15, 16, 17
B	AMP, LIN	10
C	AMP, CHL, LIN, CEP	18, 19, 20, 21, 22, 23, 24, 25, 26, 27

AMP: Ampicillin, CHL: Chloramphenicol, SXT: Trimethoprim-sulfamethoxazole, LIN: Lincomycin, LSP: Linco-spectin, OXI: Oxytetracycline, CEP: Cephalothin.

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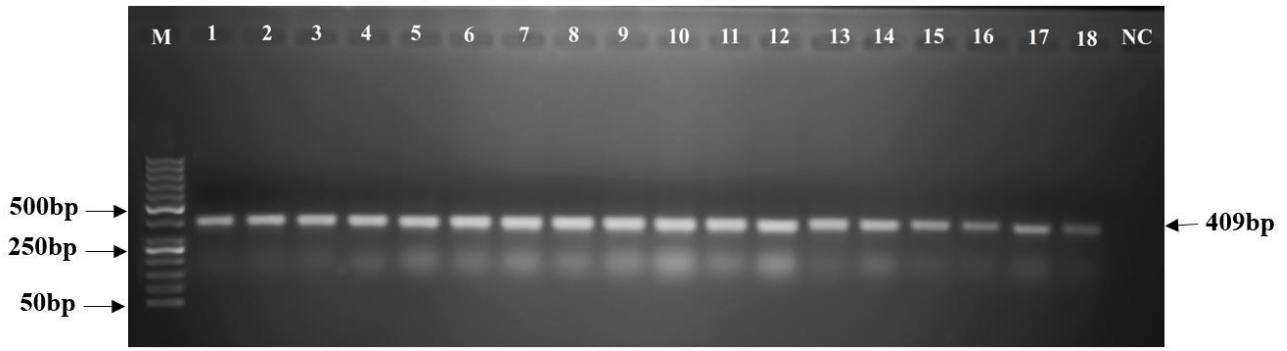

Figure 1. 16S rRNA PCR amplification analysis on gel (1.8%) electrophoresis to verify *Y. ruckeri* isolates from Rainbow trout. Lane M: 50 bp DNA ladder; lanes 1-17: Positive isolates; lane 18: Positive control *Y. ruckeri* ATCC 29473; lane NC: Negative control (distilled water).

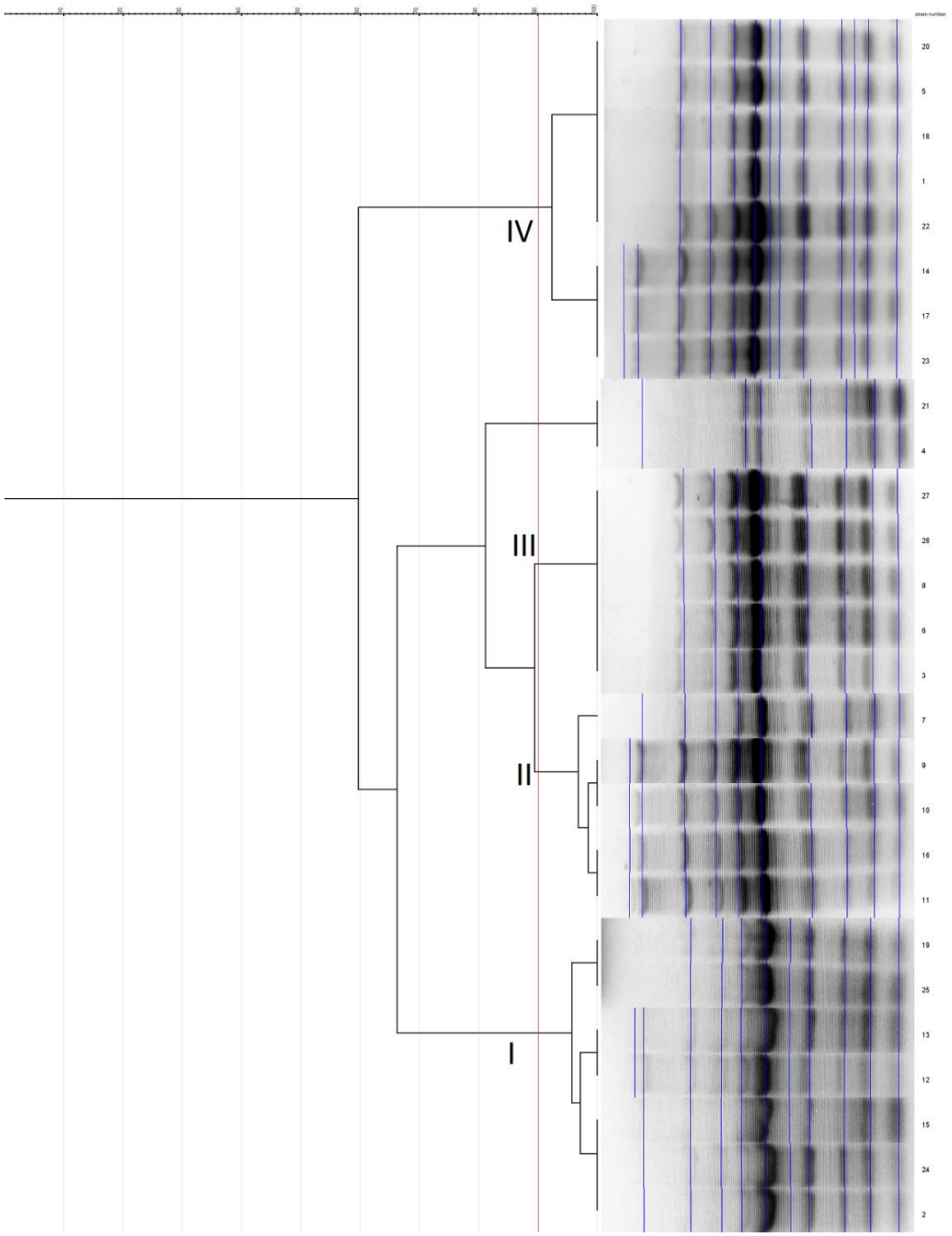

Figure 2. Dendrogram of REP-PCR for discrimination of 27 *Y. ruckeri* isolates from Rainbow trout farms with 4 clusters. The size of the bands is between 150 and 2700 bps. Samples 2, 24, 15, 12, 13, 25, 19 in first cluster, samples 11, 16, 10, 9, 7 in second cluster, samples 3, 6, 8, 26, 27, 4, 21 in third cluster, samples 23, 17, 14, 22, 1, 18, 5, 20 in the fourth cluster.

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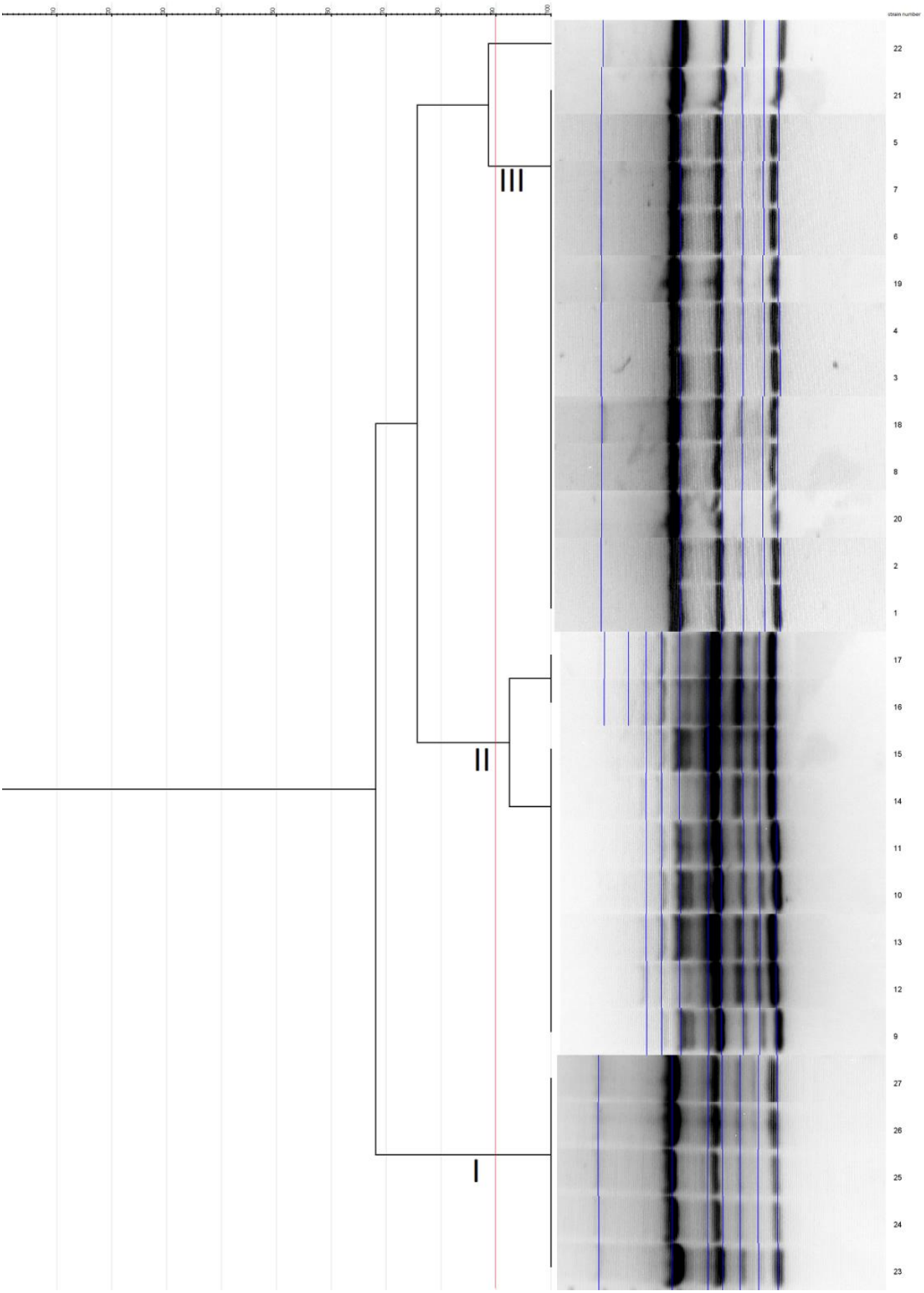

Figure 3. Dendrogram of RAPD-PCR for discrimination of 27 *Y. ruckeri* isolates from Rainbow trout farms with 3 clusters and one singleton. The size of the bands was between 300 and 2300 bps. Samples 24, 23, 25, 26, 27 in first cluster, samples 9, 12, 13, 10, 11, 14, 15, 16, 17 in second cluster, samples 1, 2, 20, 8, 18, 3, 4, 19, 6, 7, 5, 21 in third cluster, and sample 22 is a single tone.

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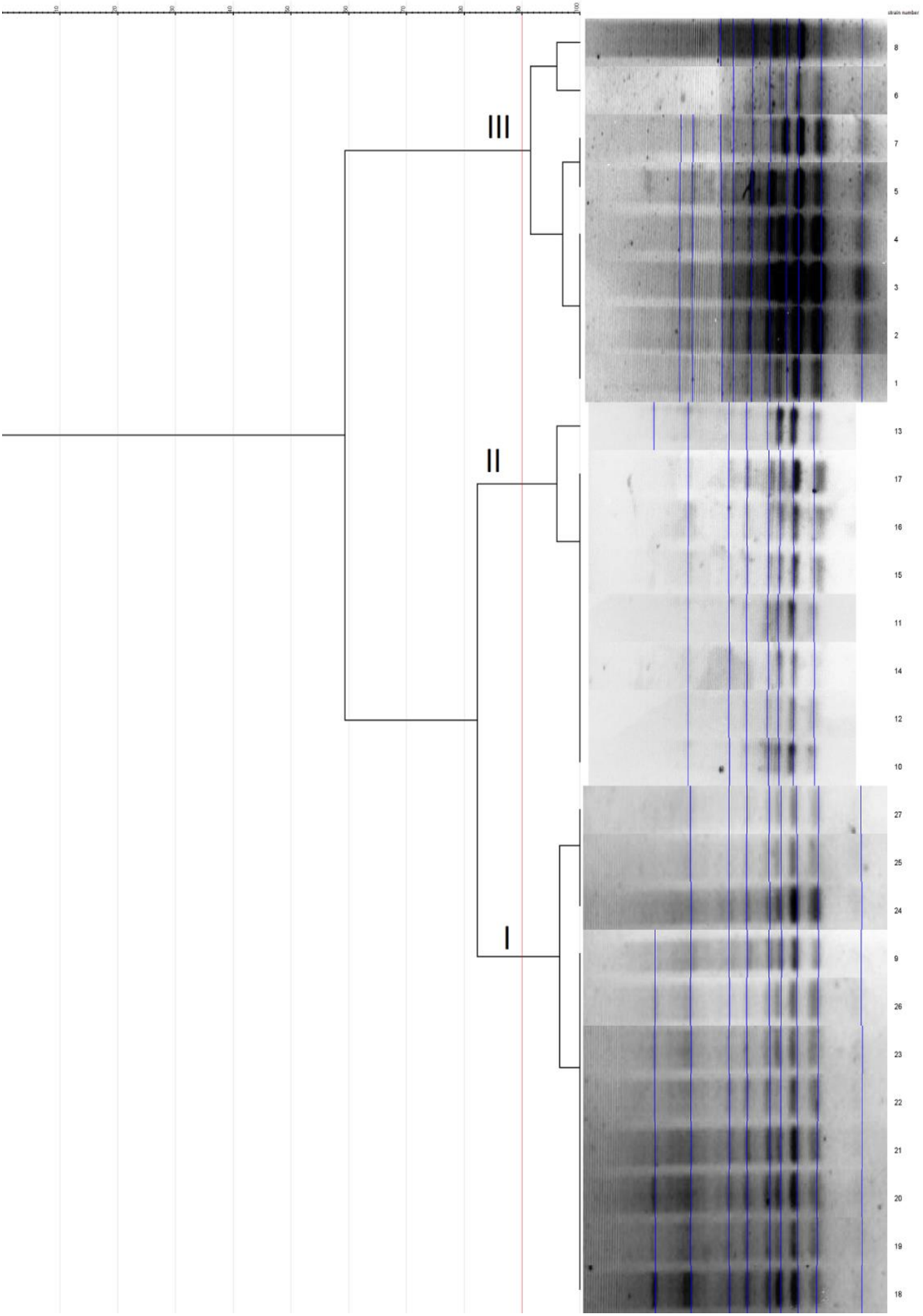

Figure 4. Dendrogram of ERIC PCR for discrimination of 27 *Y. ruckeri* isolates from Rainbow trout farms with 4 clusters. The size of the bands was between 150 and 1600bp. Samples 18, 19, 20, 21, 22, 23, 26, 9, 24, 25, 27 in first cluster, samples 10, 12, 14, 11, 15, 16, 17, 13 in second cluster, samples 1, 2, 3, 4, 5, 7, 6, 8 in third cluster.

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DISCUSSION

The emergence of resistant isolates is a worldwide concern because it restricts treatment during farm outbreaks leading to increased mortality, and consequently financial problems. The obtained results of the current study indicated the highest sensitivity to antibiotic activity for enrofloxacin and ciprofloxacin (100%) similar to previous studies (Ture and Alp, 2016; Dinçtürk and Tanrıkul, 2021). Moreover, 25.9% of isolates were resistant to sulfamethoxazole-trimethoprim (SXT), which was higher than other reports (9-13%) by Ture and Alp (2016) and Duman et al. (2017). Since SXT is a drug choice in ERM outbreaks to treat bacterial infections in the sampling fish farms, a higher rate of SXT resistance than enrofloxacin was expected.

The resistance to tetracycline and its diversities in fish farms were observed in various studies (Miller and Harbottle, 2018; Santos and Ramos, 2018; Preena et al., 2020). In the current survey, 29.6% of isolates were resistant to oxytetracycline similar to the findings of Ture and Alp (2016), and Delalay et al. (2020). The efflux pumps play a significant role in resistance to the tetracycline issue. Two *tetA* and *tetB* genes are responsible for encoding. Moreover, isolates of the present study exhibited 100%, 62.9%, and 25.9% phenotype resistance against lincomycin, chloramphenicol, and linco-spectin. Thus, antimicrobial stewardship is needed to restrict using tetracycline and diversities to avoid the rate of resistant genes transferring among bacteria in the environment.

Although β-lactamase genes have been detected on the genome of *Y. ruckeri*, evidence demonstrates the expression of these genes is not at a high level (Kumar et al., 2015). However, in the present study, resistance against ampicillin and cephalothin were 100% and 77%, respectively. Likewise, earlier surveys reported these results (Ture and Alp, 2016; Dinçtürk and Tanrıkul, 2021).

The Simpson's diversity index (DI) in the current study indicated the highest power of diversity for REP-PCR (DI = 0.91). On the contrary, a previous study reported a higher discrimination power for ERIC than REP-PCR (Huang et al., 2013). Possible explanations for this contradiction include primer sequences and PCR conditions. Another study using four typing methods, including ERIC, PFGE, MLSA, and 16S rRNA gene sequencing within 60 *Yersinia* species revealed that ERIC, 16s rRNA gene sequencing, and MLSA divided most of *Yersinia* species into the same species-specific clusters while PFGE could not (Souza et al., 2010). Therefore, ERIC is considered a valuable method for identification and discrimination for being a less expensive, easier, and faster method than 16S rRNA and MLSA (Sedighi et al., 2020). The RAPD has shown the lowest diversity index (D = 0.72) in the current study, compared to ERIC and REP-PCR methods (D = 0.83 and 0.91, respectively). It is plausible that several limitations could affect the results obtained using a single primer in RAPD, or using a few numbers of samples. Although antimicrobial susceptibility profiling as a phenotypic method is not as accurate as molecular methods, combining these may improve the diversity index. For example, the prior survey revealed that a combination of REP-PCR and OMPs typing results elevated the diversity index to 0.90, while individual format was 0.35 for REP-PCR and 0.76 for OMPs typing (Bastardo et al., 2012).

CONCLUSION

In conclusion, the results of this study showed that the REP-PCR method is an efficient method for the discrimination of *Yersinia ruckeri* in epidemiological studies. On the other hand, according to identified isolates as multi-drug resistant, it is necessary to control using antibiotics.

DECLARATION

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Competing interests

The authors declare no conflict of interest.

Consent to publish

All authors agreed to publish the article.

Authors' contribution

ARZ and FA performed a PCR experiment and wrote the draft of the article. ARZ prepared a dendrogram of typing techniques, propounded the idea, and led the group. HN did the susceptibility testing of isolates. MR and HS edited the

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draft of the article and contributed to the analysis of dendrograms. All authors checked the article's final draft before submission to the World`s Veterinary Journal.

Ethical considerations

All the authors have checked ethical issues: plagiarism, consent to publish, misconduct, data fabrication and falsification, double publication and submission, and redundancy.

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