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DIAGNOSTICS AND ANTIBIOTIC RESISTANCE OF YERSINIA RUCKERI STRAINS ISOLATED FROM TROUT FISH FARMS IN BULGARIA

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ABSTRACT

The study included 12 *Yersinia ruckeri* isolates from rainbow trout with symptoms of yersiniosis. Phenotypic and genotypic analysis of the isolates were performed by conventional microbiological, biochemical and molecular methods. Identification was confirmed by PCR using *Y. ruckeri*-specific primers and antibiotic resistance profiles were determined. Some isolates were found to be resistant to florfenicol, erythromycin, and oxytetracycline, which are licensed for use in fish farms. The obtained results will serve as basis for development of a local diagnostic scheme and strategy to fight against *Yersinia ruckeri.*

INTRODUCATION

Bacterial diseases constitute an important epidemiological factor, as they account for serious losses in rainbow trout farms. Yersiniosis, or the so-called enteric red mouth disease (ERM), is an infectious disease of salmonid fish, eels, sand sole, sturgeon, and turbots, caused by *Yersinia ruckeri* (*Enterobacteriaceae*) as the etiological agent. The pathogen was first isolated from trout in the Hagerman Valley, USA, in 1950 (Rucker, 1966). In rainbow trout, acute infections caused by the Hagerman strain are highly demonstrable and severe, and are defined as classical enteric red mouth disease. *Yersinia ruckeri* (*Y. ruckeri*) infection leads to general haemorrhagic septicemia. The clinical manifestation includes exophthalmos and petechial haemorrhages in the eyes and oral cavity (Roberts, 1983). At present, it can be found in fish populations in Europe, North and South America, Australia and New Zealand.

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On the basis of heat stable antigens (lipopolysaccharide), *Y ruckeri* can be divided into four major serotypes: O1, O2, O3 and O4 (Romalde *et al*., 1993). Strains of *Y. ruckeri* can be grouped into clonal types on the basis of biotype, serotype and outer membrane protein types (Davies and Frerichs, 1989; Davies, 1990; Davies, 1991a and Davies, 1991b).

Most European strains of *Y. ruckeri* including the virulent 'Hagerman' strain belong to serogroup O1. On the basis of motility and Tween 80 hydrolysis, two distinct phenotypes are recognised (Davies and Frerichs, 1989). Biotype 1 strains are motile and hydrolyse Tween 80 while biotype 2 is negative for both traits. Disease control includes vaccination and administration of antimicrobial agents, primarily quinolones as oxolinic acid or flumequine (Grave *et al*., 1996; Ledo *et al*., 1987; Rodgers and Austin, 1983 and Schmidt *et al*., 2000). The aim of the present research was: 1) to compare *Y. ruckeri* isolates from different farms in Bulgaria; 2) to compare different methods used for their identification; and 3) to assess their antibiotic sensitivity to nine antimicrobial agents.

MATERIALS AND METHODS

Isolation and identification of *Y. ruckeri* Twelve *Y. ruckeri* isolates (numbered n°1 to 12) were used. They were isolated from rainbow trout bred in fish farms, with symptoms of yersiniosis. The isolates were obtained over a period of 2 years. Samples for bacteriological analysis were collected from the intestines, spleen, kidney, heart, and liver onto Tryptic Soy Agar (TSA) (Difco, Germany), *Yersinia* isolation agar (YIA) (Himedia, India), McConkey (Biomerieux, France) and Shotts-Waltman (SW) (Oxoid, Switzerland) culture media and the cultures were incubated at 25°C for two days. Suspected *Y. ruckeri* colonies were examined by Gram staining and then pure cultures were cultivated on Columbia blood agar (Biolab). For further identification of *Y. ruckeri* suspected isolates, specific biochemical reactions were used: Urease, Citrate, Indole, Methyl Red, Voges Proskauer, Oxidase, Catalase, Lysine, Decarboxylase, ONPG, Arginine dihydrolase, Ornithine Decarboxylase, Tryptophan Deaminase and Lactose, Glucose, Sucrose, Maltose, Mannitol, Melibiose, Arabinose, Rhamnose, Inositol carbonhydrate fermentation tests, nitrate reduction, and Tween 80 hydrolysis test (Davies and Frerichs, 1989 and Ross *et al*., 1966). The isolates were confirmed as *Y. ruckeri* and distinguished from *Hafnia alvei* by the Micronaut automatic identification system for quick identification (Merlin Diagnostika GmbH, Germany), Plate E (E2-510-400).

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from pure culture with Bacteria genomic DNA Fast Mini Kit (Geneshun Biotech Co., China) according to the manufacturer's instructions. The quality and quantity of the extracted DNA was measured by DNA/RNA calculator Gene Quant II (Pharmacia Biotech, UK). *Y. ruckeri* was confirmed by polymerase chain reaction using specific primers YER8 (5'-GCGAGGAGGAAGGGTTAAGTG-3') and YER10 (5'-GAAGGCACCAAGGCATCTCTG-3') targeting a non-conservative region of the 16S rRNA gene sequence of *Y. ruckeri* (Fernandez *et al*., 2004). The reaction mixture included 10 pmol/µL of each primers, 2.0 µL of extracted DNA, 21.0 µL of distilled water, and (ТОВА СА ЛИОФИЛИЗИРАНИ ПЕРЛИ И КЪМ ТЯХ СЕ ДОБАВЯТ КОМПОНЕНТИТЕ-ИЗРЕЧЕНИЕТО ТРЯБВА ДА ГО ПОЯСНЯВА-СЕГА ИЗЛИЗА ЧЕ И ТОВА СЕ ДОБАВЯы А ТО Е ГОТОВО И КЪМ НЕГО СЕ ДОБАВЯТ) puReTaq Ready-To-Go PCR Beads (GE Healthcare, UK). Negative control consist 10 pmol/µL of each primer and 23.0 µL of distilled water added to puReTaq Ready-To-Go PCR Beads (GE Healthcare, UK)

PCR conditions were as follows: initial denaturation at 95 °C/5 min then 25 cycles of: denaturation at 95 °C/1 min, annealing at 60 °C/1 min, extension at 72 °C/1 min; and a final extension step at 72 °C/5 min (Gibello *et al*., 1999). Amplification was checked by running gel electrophoresis of the PCR products in a 2 % agarose (Geneshun Biotech) gel, at 120 V for 30 min. Staining was done with 0.005 % added GoldView (Geneshun Biotech Co., China).

Specificity of the PCR reaction

To determine the specificity of the primers used in the PCR reaction, DNA obtained from *Aeromonas hydrophila* (clinical isolate), *Hafnia alvei* (clinical isolate) and *E.coli 0:157* (reference strain) were also analyzed.

Antibiotic susceptibility

The antibiotic susceptibility of the isolates to nine common antimicrobial agents (identified in table 2) was assayed by the disc diffusion method (Bauer *et al*., 1966) in accordance with the CLSI (Clinical Standards Institute) recommendations.

RESULTS

Diagnostics of yersiniosis is based on observation of clinical symptoms and isolation of pure bacterial culture from the anterior region of the liver or spleen. Typical external symptoms of yersiniosis, e.g. darkening, in appetence and sluggish movements and mortality, have been reported in all fish from which *Y. ruckeri* strains have been isolated. Similar pathologic anatomical changes were observed in the rainbow trout targeted in this study: pale gills, haemorrhages at the basis of the ventral and pectoral fins, the eyes and the anus. Typical yersiniosis (red mouth) signs were hemorrhages on the upper and lower jaw and the tongue (*Fig. 1b*), and petechial hemorrhages in the liver and intestines (*Fig. 1a*). In all the studied fish, the spleen and kidneys were dark and visibly enlarged, and yellow mucous fluid was accumulated in the intestine. The phenotypic characterization of the isolates revealed Gram-negative rods with rounded ends, 0.5–0.8 µm × 1.0–3.0 µm in size, motile, non-spore-forming and without capsules. Like all *Enterobacteriaceae* representatives, the isolates were found to be glucose-fermenting, oxydase-negative, nitrate-reducing, and non-hemolytic (Table 1).

Inoculations from internal organs of fish onto TSA, YIA, McConkey and SW culture media resulted in growth of *Y. ruckeri* which produced small, round, white and cream color colonies of 1-2 cm diameter on TSA following 1-2 days of inoculation at 25 °C, color less colony on McConkey agar following 1-2 days of inoculation and light green color colonies following 3-4 days of inoculation on SW. On YIA after incubation at 25°C for 48h, *Y. ruckeri* isolates grew well and produced small pink entire edged with a low convex profile colony which are 1-2 mm in diameter. On Blood agar after incubation at 25°C for 48 hours, *Y. ruckeri* isolates produced off-white, opaque with a marked bull's eye colonies which are approximately 2-3 mm in diameter, non hemolytic, smooth, entire edged with a low convex profile.

The biochemical characterization based on lysine decarboxylase reaction; urease reaction; arginine and ornithine decarboxylase reactions; ramnose, sucrose, xylose, sorbitol fermentation etc. by the Micronaut system confirmed 11 isolates as *Y. ruckeri* (with 97 % to 100 % probability*)* and 1 isolate as *Y. kristensenii* (with 74% probability) (Table 1). All the studied *Y. ruckeri* isolates were genotypically confirmed by PCR using a pair of primers targeting a specific 575 pb fragment (Fig. 2). The isolate identified as *Y. kristensenii* based on biochemical characteristics was also PCR positive. Based on the clinical manifestation and the PCR results, the isolate biochemically identified as *Y. kristensenii* was considered to be *Y. ruckeri*.. No unspecific amplification was obtained with a group of control strains including *Aeromonas hydrophila* (clinical isolate), *Hafnia alvei* (clinical isolate), and

Table 1.

Isolate number	ODC	URE	ADH	ESC	LDH	TDA	CIT	ONPG	MAL	IND	XYLF	Identified as (relative probability %)
1	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (100)
2	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (100)
3	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (100)
4	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (100)
5	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (100)
6	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (100)
7	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (99.9)
8	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (99.34)
9	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (99.32)
10	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (97.87)
11	+	-	-	-	+	+	-	+	-	-	-	*Y. ruckeri* (97.67)
12	+	+/-	-	-	-	-	-	+/-	-	-	-	*Y. kristensenii* (74)

* ODC – ornithindecarboxylase, URE-urease, ADH-arginindihydrolase, ESC -esculin, LDH-lysinedecarboxylase, TDA- tryptophandesaminase, CIT - citrate, ONPG-0-Nitropheny-ß-galactosidasel, MAL- malonate, IND-indole, XYLF-xylose

Table 2.

Isolate *Y. ruckeri*	OA 2µg	OT 30µg	R, I, S Nx 30µg	(IZD mm) E 15µg	A 10µg	ST 23/1.25µg	Ox 1µg	F 30µg	CeQ 1µg
1	S(30)	R(10)	S (34)	S(30)	R(3)	S(28)	R(2)	I(20)	S(30)
2	S(32)	S(32)	S (33)	R(2)	R(2)	S(29)	R(2)	S(29)	R (2)
3	S(30)	S(30)	S(34)	S(27)	R(3)	S(28)	R(2)	S(28)	S (31)
4	S(31)	S(34)	S(34)	S(27)	R(2)	S(27)	R(3)	I(22)	S(29)
5	S(30)	S(35)	S(32)	R(3)	R(3)	S(28)	R(2)	S(27)	S(30)
6	S(30)	S(34)	S(33)	R(7)	R(3)	S(28)	R(3)	S(30)	S(30)
7	S(30)	S(32)	S(34)	R(8)	R(2)	S(28)	R(3)	S(27)	S(28)
8	S(31)	R(10)	S(34)	R(10)	R(2)	S(29)	R(2)	S(29)	S(30)
9	S(30)	S(30)	S(33)	S(29)	R(2)	S(28)	R(2)	R(9)	R(5)
10	S(30)	S(30)	S(32)	R(10)	R(1)	S(30)	R(1)	S(28)	S(30)
11	S(31)	S(30)	S(33)	R(10)	R(2)	S(29)	R(1)	S(29)	R(8)
12	S(30)	I (15)	S(34)	S(28)	R(2)	S(28)	R(2)	I(20)	S(31)

E.coli 0:157 (reference strain). By the disk diffusion method, all clinical isolates showed one and the same antimicrobial susceptibility pattern against nalidixic acid, oxolinic acid, sulfamethoxazone/trimetoprim, with inhibition zone diameter (IZD) values of 30-32, 32-34, and 27-30 mm, respectively. Some of the tested isolates shoed resistance to Cefquinom (IZD values of 2, 5, 8 mm), florfenicol (IZD values of 20, 22, 9 and 20 mm), erythromycin (IZD values of 2, 3, 7, 8, 10, 10, 10 mm), and oxytetracycline (IZD values of 10, 10, and 15 mm), which are licensed for use in fish farms in Bulgaria (Table 2). Two *Y. ruckeri* isolates shoed resistance to florfenicol and oxytetracycline. One isolate was resistant to florfenicol, erythromycin and oxytetracycline. None of the isolates were resistant to all nine tested antibacterial substances. All isolates shoed resistance to Ampicillin and Oxacillin (Table 2).

DISCUSSION

Cold-water aquaculture species, such as trout, are very susceptible to enteric red mouth disease (Furones *et al*., 1993). The infection is mainly observed in young rainbow trout. In addition, all wild and cultured salmonids are susceptible to disease (Horne and Barnes, 1999). Factors causing stress in fish such as excessive stocking, pollution, oxygen depletion play a key role in the outbreak of the disease. The infection spreads from fish to fish via water. Severe epidemics are observed at temperatures between 15-18ºC. The disease generally appears in per acute and acute forms in young fish during spring months when sudden increases in water temperatures appear; and in chronic form in fish of one year old during autumn months when water temperatures fall (Busch, 1978). The causative agent colonized the small intestine, spleen and liver of fish without showing any clinical signs until after 60-65 days of infection (Busch, 1982). Yersiniosis is now found in north-west USA, Canada, Europe, Australia, north Africa and Asia. The causative agent is spread by asymptomatic carriers to California, Nevada, Colorado, and a number of European countries (Furones *et al*., 1993).

This wide spread of *Y. ruckeri* is thought to result from salmonid aquaculture trade. It was first believed that the pathogen was spread by asymptomatic carriers and spawn, but since *Y. ruckeri* was isolated from mammals, it has been suggested that wild animals such as birds, free-living fish, amphibians, and even human, could serve as vectors (Willumsen, 1989). Sauter *et al.* (1985) isolated *Y. ruckeri* from salmon spawn (*Oncorhynchus tshawytscha)* and speculated that vertical transmission may also be possible, although evidence in support of this hypothesis has not been found yet. *Y. ruckeri* produces various toxins, e.g. haemolysins, endotoxins and cytokinins (Aussel *et al*., 2000; Fernandez *et al*., 2004; Romalde and Toranzo, 1993 and Secades and Guijjarro, 1999), but little is still known about its pathogenicity factors. Like the other Enterobacteria, *Y. ruckeri* is a glucose-fermenting, catalase-positive, oxidase-negative, and nitrate-reducing microorganism (Ross *et al*., 1966). *Yersinia ruckeri* is distinguished from the other members of the *Yersinia* genus by its lysine decarboxylase activity (Bottone *et al*., 2005). The *Y. ruckeri* strains have relatively homogenous biochemical characteristics, although they show some variations in the Voges–Proskauer (VP) reaction,

sorbitol fermentation and Tween 80 hydrolysis. The conventional biochemical methods used as a "gold standard" in the identification of *Yersinia spp*. may prove insufficiently accurate in the identification of some isolates (Ibrahim *et al*., 1993). Based on its biochemical characteristics, one of our isolates was identified as *Y. kristensenii* (TDA-; URE+; ONPG-; CIT-). Biochemical tests do not always give identical reactions for different *Y. ruckeri* isolates (Austin *et al*., 2003; Degrandis *et al*., 1988 and Ross *et al*., 1966). If only phenotypic characteristics are taken into account, *Y. ruckeri* can be misidentified as different representatives of other taxonomic groups, such as *Serratia*, *Hafnia*, or *Salmonella* (Austin *et al*., 2003 and Llewellyn, 1980). Despite the fact that repeated subculturing may lead to reduced virulence and loss or change of some biochemical characteristics (Thyssen *et al*., 1998), the traditional diagnostic characteristics based on phenotype are still considered effective in the diagnostics of infections caused by *Y. ruckeri* (Austin *et al*., 2003). *Y. ruckeri* can also be identified with automated systems for fast identification (Coquet *et al*., 2002).

The interpretation of the results obtained from automated systems for fast identification like Micronaut and API 20E is still problematic when bt 1, bt 2 and new biogroups are analyzed. In particular, the profile could be mistaken with that of *Hafnia alvei*, which is another *Enterobacteriaceae* representative (Austin *et al*., 2003). *Hafnia alvei* is similar morphologically and serologically to *Y. ruckeri* but can be readily differentiated by phenotype. Key differential tests are motility at 37°C, citrate use, gluconate oxidation, or fermentation of rhamnose and xylose (Carson and Wilson, 2009). The main characteristics used for distinguishing between bt 1 and bt 2 are motility and Tween 80 hydrolysis (Davies and Frerichs, 1989). Biotype 2 consists of non-motile, Tween 80 hydrolysis negative strains, most of which have been found only in the United Kingdom and Norway and as an emerging type in Australia (Carson and Wilson, 2009). In recent years, bt 2 *Y. ruckeri* isolates have been reported to cause disease in prevaccinated rainbow trout (Austin *et al*., 2003). The bacteria which incur losses in vaccinated aquaculture fish differ from the earlier definition of bt 2 in that they are non-motile and VP positive. The VP positive isolates now constitute a new biogroup called "EX5". All the isolates in our study were identified as bt 1: motile, positive for Tween 80 hydrolysis and VP negative.

The lack of universal serum against the different *Y. ruckeri* serotypes and biotypes renders serology inapplicable in many laboratories. The motile bt 1 representatives are peritrichous (Davies and Frerichs, 1989). The bacteria grow in a wide temperature range, with an optimum at 28 °C (Stevenson *et al*., 1993). Studies (Austin *et al*., 1982) on *Y. ruckeri* have found that changes in the contents of the nutrient medium may result in altered external morphology of the cells. For example, addition of 3 % (w/v) NaCl to TCA brought about significant differences in the cell size, from 0.5 μm to 4 μm. *Y. ruckeri* can be isolated after cultivation in culture media such as TSA, BHIA, ROD and SW, by incubating for 48 hours at 22-25°C. The comparison of SW with the other culture media suggested that there were no significant differences between them and SW medium can therefore be used for epidemiological studies owing to its advantages such as high sensitivity and shorter time requirement (Austin *et al*. 2007). Characteristic colonies in blue-green color were obtained following the inoculations onto SW culture media. However, specific colonies were only distinctive in 72- 96 hours, not in 24-48 hours as mentioned by the above researchers. Some authors comment appearing of distinctive colony on SW medium in 96-120h after inoculation (Şeker *et al*., 2011). According to Seker *et al.* (2011) evaluations of ERM suspected cases should be made after checking growths on SW selective medium for one week. YIA medium has been developed for selective isolation of *Yersinia* species and preliminary differentiation of *Y. enterocolitica* from human and animal intestinal contents (Wauters, 1973). The medium is recommended by the ISO Committee for identification of *Yersinia* species from foods (International Organization for Standardization (ISO), 1994 Draft ISO/DIS 10273). Within 24 h of incubation at 28°C or after 48h at 25°C, *Y. ruckeri* exhibits good-luxuriant growth. The comparison of YIA with SW medium suggested that YAI can be used as selective medium for diagnosis of *Y. ruckeri* holding out shorter time for isolation of bacterium. The routine microbiological methods for cultivation and identification are time-consuming, which may delay the diagnosis of the disease. Delayed diagnosis increases the losses and the mortality, and adds to the owner's pessimism, which negatively affects the whole branch.

The loss of appetite as the disease progresses, hinders the oral treatment with chemotherapeutics. In a recent phylogenetic analysis of the *Yersinia* genus, based on 16S rRNA sequencing, Ibrahim *et al.* (1993) revealed that the genus forms a subgroup consisting of 5 closely linked subspecies in the *Enterobacteriaceae* family, *Y. ruckeri* constituting a separate subspecies. This indicates that specific oligonucleotides based on differences in 16S rRNA sequences can be employed in the diagnostics of *Y. ruckeri* by PCR (Mcintosh *et al*., 1996; Saulnier and Kinkelin, 1997; Urdaci *et al*., 1998 and Zlotkin *et al*., 1998). Specific DNA amplification by PCR is a highly sensitive and specific method; it is an alternative approach for detection and identification of bacteria of various origins (Gibello *et al*., 1999 and Rys and Persing, 1993). Argenton *et al.* (1996) described a PCR based method for diagnostics of *Y. ruckeri*. A PCR assay for detection of *Y. ruckeri* in different tissues of infected trout was developed based on amplification of non-conservative rDNA regions (Gibello *et al*., 1999). Some authors have proposed that early diagnostics of yersiniosis may be achieved within 12 h by PCR analysis of a small amount of blood from the heart of living fish (Altinok *et al*., 2001 and Şeker *et al*., 2011).

The PCR method has been proved useful for detection of asymptomatic carriers which are difficult to identify by means of routine bacteriological techniques (Busch and Lingg, 1975). The detection of asymptomatic carriers is of special importance for preventing the disease transmission and the spread (Leclercq *et al*., 1996). PCR has gained wide popularity as an alternative to traditional identification methods (White *et al*., 1992). Using PCR, we confirmed all our isolates to be *Yersinia ruckeri.* We also confirmed the specificity of the chosen primers for detection of the infectious agent. The amplification of DNA from all the 12 studied isolates yielded the anticipated 575-bp amplicon. Amplification of DNA from none of the other species used as controls (*Aeromonas hydrophila*, *Hafnia alvei* and *Escherichia coli*) yielded this

PCR product when the same primers (YER8 and YER10) were used (Fig. 3). Similar results have been reported by other authors (Altinok *et al*., 2001; Gibello *et al*., 1999; Kirkan *et al*., 2006; Onuk *et al*., 2011; Şeker *et al*., 2011 and Sirvas-Cornejo te al., 2001). The primer set YER8-YER10 gave an unique and specific amplification band with a 575-bp lenght at an annealing temperature of 60°C with Y. Ruckeri strains only (Gibello *et al*., 1999). Antimicrobial agents are often used in fish in the treatment of infections caused by *Y. ruckeri.* Rucker (1966) described treatment with Sulphamethazine for 5 days, followed by Chloramphenicol or Oxytetracycline for 3 days. Sulphonamide therapy has been successful in experimental and natural infections (Bullock *et al*., 1983). Rodgers and Austin (1983) used oxolinic acid for prophylaxis and treatment of enteric redmouth disease in trout. Chloramphenicol or oxytetracycline are used for disease control (Rodgers, 1990). In our country Chloramphenicol was permitted for use in aquacultures till 1994. Misuse of antimicrobials results in emergence of dangerous resistant strains (Coquet *et al*., 2002).

Moreover, when bacteria are subjected to improper or varying drug concentrations, the prescribed treatment period is not completed, or treatment with the same drug is repeated shortly, drug resistant strains are sure to be selected (Rodgers, 2001). The same author also explains how bt 1 isolates acquire resistance to oxolinic acid, oxytetracycline and sulphonamide as a result of frequent use of antimicrobials. Althouth *Y. ruckeri* is sensitive to a number of antibiotics, there is an ever increasing number of reports about acquired resistance of *Y. ruckeri* strains to different antimicrobial agents. Post (1984) warned about the full resistance of some isolates from the USA to therapeutic doses of Sulphamerazine, as well as of Oxytetracycline. Resistance to tetracyclines and sulphonamides has also been reported (Degrandis and Stevenson, 1982). As seen from Table 3, our study identified resistance to cefquinom, oxytetracycline, erythromycin and florphenicol. Nowadays the most used antibiotics against bacterial diseases in aquacultures in Bulgaria are oxytetracicline and florfenicol. Detected bacterial resistance to these antibiotics is annoying and determine the use of the quinolones as the next final level of defense against *Y. ruckeri.* Quinolones are very useful antimicrobial agents that have proven to be helpful in different clinical contexts in both human and veterinary medicine (Fernandez *et al*., 2004).

Despite the isolation of quinolone-resistant clinical strains of *Y. ruckeri* is unusual (Oraic *et al*., 2002 and Schmidt *et al*., 2000) application of quinolones should be limited and they must be used in emergency case only. Antibiotics used in human medicine, such as oxytetracycline, sulfamerazine, and ormetoprim, are employed for treatment of infections in economically important fish species, e.g. salmon, trout, etc. The most frequent infections treated in fish are ulcers, sepsis, and enteritis. The bacteria that cause these infections, including *Y. ruckeri*, belong to bacterial families which include human pathogens. It is very probable that transfer of drug resistance could occur. Resistance may be transferred even when the treatment has finished before the fish is sold. That is why, from an environmental and health perspective, the use of antibiotics in fisheries should be reduced, and if required, it should be done under strict veterinary supervision.

In conclusion, it may say that *Yersinia ruckeri* strains biotype1 were isolated from 4 Bulgarian fish farms in which rainbow trout with clinical symptoms of yersiniosis were detected. The isolates were phenotypically characterized, and resistance to oxytetracycline and florphenicol was observed. It was found that *Yersinia* isolation on agar is suitable for isolation of *Y. ruckeri* and can be used for diagnosis of yersiniosis. PCR based method for rapid diagnostics of *Y. ruckeri* was introduced for the first time in Bulgaria as routine laboratory method. Used PCR assay was confirmed as successful and strictly sensitive in the diagnostics of *Yersinia ruckeri* in naturally infected trout.

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