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NOTE

Analysis of *Yersinia ruckeri* strains isolated from trout farms in northwest Germany

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ABSTRACT: Enteric redmouth disease (ERM), caused by *Yersinia ruckeri*, is among the most important infectious diseases in rainbow trout *Oncorhynchus mykiss* aquaculture in Europe. Our aim was to analyse the persistence of *Y. ruckeri* strains in trout farms in northwest Germany and their dissemination between farms based on a detailed molecular and phenotypical characterisation scheme. The data on identification and characterisation of *Y. ruckeri* strains and examining the distribution of these strains in the field could serve as a basis for preventive disease monitoring plans. During the observation period from June 2011 until June 2012, we collected 48 *Y. ruckeri* isolates from 12 different rainbow trout hatcheries. In total, 44 (91.7%) of the isolates were non-motile; in particular, all isolates recovered during the sampling period in winter and early spring were non-motile. In several trout farms, characteristic farm-specific *Y. ruckeri* isolates from particular typing groups were isolated throughout the year, while in other farms, which had a trading relationship between each other, ERM outbreaks were caused by *Y. ruckeri* from the same typing group. Our data indicate that in some farms, the causative *Y. ruckeri* strains persisted in the respective trout farm. The presence of *Y. ruckeri* from the same typing group in farms with a trading relationship indicates a dissemination of the infection between the farms.

KEY WORDS: Epidemiology · Enteric redmouth disease · Rainbow trout · *Oncorhynchus mykiss*

INTRODUCTION

Enteric redmouth disease (ERM) is a serious disease of farmed rainbow trout *Oncorhynchus mykiss* that causes economic losses worldwide. The aetiologic agent of ERM, the enterobacterium *Yersinia ruckeri*, has been isolated from various fish species, including rainbow trout, lake trout *Salvelinus namaycush*, brown trout *Salmo trutta* and Atlantic salmon *Salmo salar*, but mainly affects rainbow trout. Clinical outbreaks of ERM are characterised by haemorrhages in various tissues, particularly around the mouth, in the muscles, the peritoneum and the lower intestine. Mortality in rainbow trout farms where ERM is present can reach up to 70% of the total population (Furones et al. 1993). ERM outbreaks can be treated with antibiotics, but it is difficult to eradicate the disease from a farm completely. In the farm environment, *Y. ruckeri* can survive for several months outside the host in water, sediment or bio-

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films of fish tanks (Furones et al. 1993, Coquet et al. 2002). In addition, infected fish may become asymptomatic carriers and shedders, and may thereby infect other fish. This may happen on the same farm or on other farms when such infected fish have been moved between facilities. Hence *Y. ruckeri* can easily spread from one trout population to another and from one farm to another. In order to prevent devastating disease outbreaks and massive antibiotic treatment, appropriate vaccination and good husbandry is essential (Villumsen et al. 2014). For vaccination, ERM vaccines are mainly applied by the immersion method, which allows vaccinating large numbers of juveniles in a fast and reliable manner (Chettri et al. 2012). Nevertheless, outbreaks of ERM are still periodically observed, in particular in trout farms where *Y. ruckeri* is endemic. These outbreaks have often been linked to the presence of non-motile isolates (assigned as biotype 2, BT 2, isolates). Non-motile BT 2 isolates were found in trout populations in the UK, the European mainland (Austin et al. 2003, Fouz et al. 2006, Ström-Bestor et al. 2010) and North America (Arias et al. 2007). The first isolation from a trout population in Germany dates back to 1994 (Klein et al. 1994). Recently, 'vaccine-tolerant' BT 2 isolates of *Y. ruckeri* were recorded from ERM outbreaks in trout farms in the German federal state of North Rhine-Westphalia (NRW; D. Mock pers. obs). In order to investigate the occurrence and further spreading of these 'vaccine-tolerant' isolates, *Y. ruckeri* strains isolated from trout farms in NRW were analysed with the molecular and phenotypic characterisation scheme for *Y. ruckeri* developed by Huang et al. (2013).

MATERIALS AND METHODS

Sample collection

Rainbow trout were sampled from tanks or ponds of 12 different trout farms in NRW in 2011 to 2012 (Table 1). The farms were located at the headwaters of the Lippe, Lenne, Ruhr and Agger Rivers, which drain into the Rhine, and at the headwaters of the Rur, which drains into the Maas (Fig. 1). All farms were monitored on a regular basis by the veterinarians of the NRW fish health service.

For stocking, all farms purchased eggs from sources certified as disease-free. In 9 of the examined farms, *Yersinia ruckeri* was endemic, and in 6 farms, the bacterium caused ERM outbreaks on a regular basis. This was confirmed by isolation of *Y. ruckeri* from tissues of clinically diseased fish by the NRW fish health service. An additional 3 farms had no previous ERM disease history. Vaccines raised against motile and non-motile strains of *Y. ruckeri* were applied in 4 farms with ERM outbreaks, while trout in all other farms were not vaccinated (see Table 1). In addition to ERM, infections with *Flavobacterium psychrophilum* (causing rainbow trout fry syndrome, RFTS) and *Aeromonas salmonicida* (causing furunculosis) were also present in rainbow trout on some farms (Table 1). For treatment of clinical outbreaks of the bacterial infections, antimicrobial agents (sulphonamides/trimethoprim, amoxicillin or florfenicol) were applied in most farms, while trout in 1 farm were not treated with antimicrobial agents at all (Table 1).

Sampling was conducted during 6 campaigns from June 2011 to June 2012. The water temperature varied from 4°C in winter to 18°C in summer (Table 2). In the farms, trout with clinical signs indicating a bacterial infection were selected from ponds with clinical outbreaks of ERM, whereas trout from apparently healthy stocks were randomly sampled. An overview of the farm visits during the sampling campaigns, sampled tanks or ponds and the number of collected trout is given in Table S1 in the Supplement, available at www.int-res.com/articles/suppl/d116p243_supp.pdf.

Isolation of bacteria

Rainbow trout were transported to the laboratory alive and examined for the presence of a *Y. ruckeri* infection following the diagnostic procedures of finfish and shellfish pathogens (American Fisheries Society Fish Health Section 2014). Kidney and spleen samples were collected from trout showing clinical signs and inoculated into tryptic soy agar. *Y. ruckeri* were isolated and identified as described previously (Huang et al. 2013). Briefly, the isolates were examined by Gram staining and were biochemically characterised by API 20E tests (bioMérieux). In addition, repetitive sequence-based PCRs were performed, including BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR), $(GTG)_5$-PCR, enterobacterial repetitive intergenic consensus (ERIC) PCR and repetitive extragenic palindromic (REP) PCR, as described by Amann (2007). Moreover, pulsed-field gel electrophoresis (PFGE) of *Not*I-digested genomic DNA of the *Y. ruckeri* isolates was performed as described previously (Huang et al. 2013). Subsequently, the detailed molecular and phenotypical characterisation scheme based on the API 20E profiles and the

Table 1. Overview of investigated fish farms, river systems and disease history. ERM/bacterial diseases were diagnosed by veterinarians of the North Rhine-Westphalian fish health service and confirmed by bacterial isolation. ERM: enteric redmouth disease (agent: *Yersinia ruckeri*); RTFS: rainbow trout fry syndrome (agent: *Flavobacterium psychrophilum*); furunculosis (agent: *Aeromonas salmonicida*)

River system	Farm	Species kept	Vaccination	ERM/bacterial disease history	Antimicrobial treatment	Trading relationship with study farm(s)	Visit(s)
Lippe, Rhine	BR	Rainbow trout *Oncorhynchus mykiss*	Yes	ERM, RTFS	Sulphonamides/trimethoprim, florfenicol	No	6
	NA	Rainbow trout	No	Occasionally ERM	Sulphonamides/trimethoprim	No	1
Lenne, Ruhr, Rhine	AL	Atlantic salmon *Salmo salar*, Brook trout *Salvelinus fontinalis*, brown trout *Salmo trutta*, rainbow trout	No	Occasionally ERM, furunculosis	Sulphonamides/trimethoprim, amoxicillin	No	1
	FL	Brook trout, brown trout, rainbow trout	No	ERM, RTFS	Sulphonamides/trimethoprim, amoxicillin, florfenicol	No	3
	FR	Brook trout, brown trout, rainbow trout	No	No	Sulphonamides/trimethoprim, amoxicillin	SR	4
	SR	Brook trout, brown trout, rainbow trout	No	ERM	Sulphonamides/trimethoprim, amoxicillin	KA, PI, FR	6
Ruhr, Rhine	ST	Rainbow trout	No	Occasionally ERM	Sulphonamides/trimethoprim	No	2
Agger, Rhine	KA	Brown trout, rainbow trout	Yes	ERM, RTFS	Sulphonamides/trimethoprim, amoxicillin, florfenicol	SR, PI	6
	PI	Brook trout, brown trout, rainbow trout	Yes	ERM, RTFS	Sulphonamides/trimethoprim, amoxicillin, florfenicol	SR, KA	6
Rhine	FI	Rainbow trout	Yes	ERM, RTFS	Sulphonamides/trimethoprim, amoxicillin, florfenicol	No	3
Rur, Maas rainbow trout	MU	Atlantic salmon, brook trout, brown trout, rainbow trout	No	No	No antimicrobial treatment	MS	2
	MS	Brook trout, brown trout,	No	RTFS	Sulphonamides/trimethoprim, amoxicillin, florfenicol	MU	2

patterns received by PFGE macrorestriction analysis and the above mentioned repetitive sequence-based PCRs (Huang et al. 2013) was applied to investigate the persistence of *Y. ruckeri* isolates in the farms and their dissemination between farms.

RESULTS

Sample collection in NRW

During the field study, 12 rainbow trout hatcheries were visited in 6 sampling campaigns in different seasons between 2011 and 2012. In total, 530 rainbow trout from 91 tanks, ponds or raceways were sampled. We were able to isolate *Yersinia ruckeri* from 183 trout collected from 39 tanks or ponds in 9 trout hatcheries, while *Y. ruckeri* could not be detected in trout from 3 hatcheries (Tables 2 & S1). During the sampling period, 4 farms with a previous history of ERM outbreaks were visited 6 times. In 2 of these farms (abbreviated SR and PI, see Fig. 1), *Y. ruckeri* was isolated from 60.5% and 65.2% of the sampled trout, respectively (Table 2), while in the farms BR and KA, only 42.4% and 21.7%, respectively, of the examined trout were positive for *Y. ruckeri*.

Y. ruckeri was found in all seasons during the sampling period, but outbreaks of ERM were more frequently diagnosed in summer and early autumn when the water temperature ranged between 10 and 15°C, compared to samples taken in winter and early spring, when water temperatures were below 10°C (Table 3). In February 2012, at a water temperature of 4–5°C, the overall prevalence of *Y. ruckeri* was 25.0%, whereas in September and April, at water temperatures of 10–15 and 6–8°C, respectively, *Y. ruckeri* was detected at a prevalence of 31.6 and 31.9% (Table 3). The sharp decrease in the prevalence of *Y. ruckeri* from to

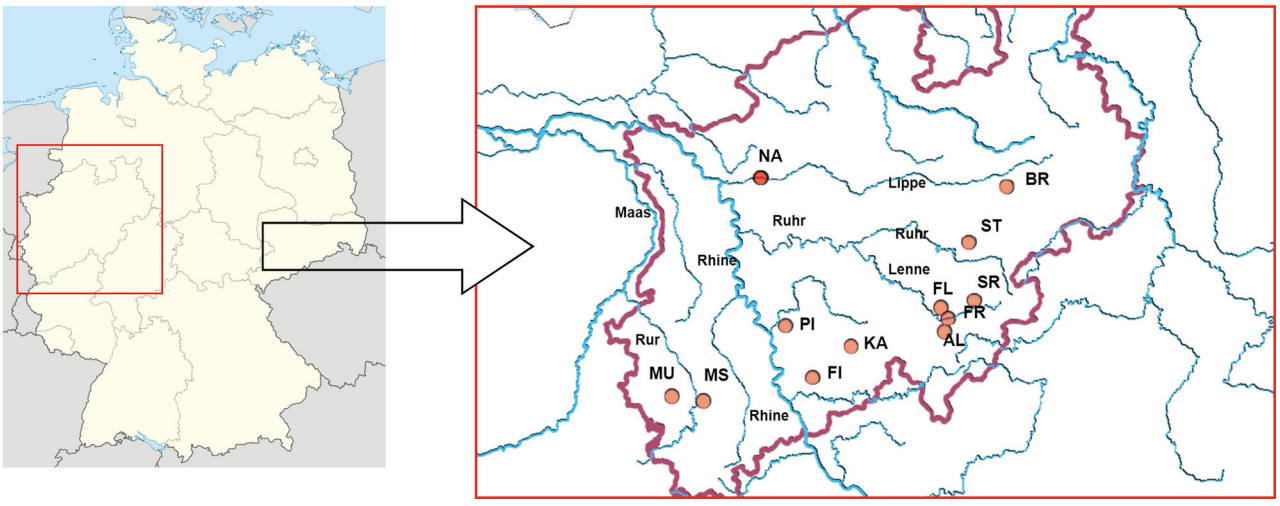

Fig. 1. Geographic origins of *Yersinia ruckeri* isolates from rainbow trout *Oncorhynchus mykiss* hatcheries in the federal state of North Rhine-Westphalia, northwest Germany. Farm locations are indicated by 2-letter abbreviations

61.9% in June 2011 to 17.3% in July 2011 resulted from a treatment of infected stocks with antibiotics after clinical outbreaks of ERM in farms BR, FL and KA. Isolates recovered in February and April were found to be non-motile in further tests.

Distribution of *Yersinia ruckeri* isolates

During this field study, a total of 48 *Y. ruckeri* isolates were obtained from 16 of the 27 typing groups (TP) described by Huang et al. (2013) (Table 3). The TPs were defined according to results obtained from biochemical (API 20 E) and molecular biological typing methods (repetitive PCR and PFGE macrorestriction analysis). In most fish farms, a specific pattern of genetic groups of *Y. ruckeri* was present, and no genetic group was detected in all 9 of the infected farms. Most isolates were non-motile; only *Y. ruckeri* isolated from diseased trout in farm FL were motile. These isolates from TP 16 were not detected in other farms included in this study and were not detected in seasons when water temperatures were lower than 10°C (Table 3).

In all farms with recurrent *Y. ruckeri* infections, farm-specific, characteristic isolates from a particular

Table 2. Rates (%, with number positive/number sampled in parentheses) of *Yersinia ruckeri* positive rainbow trout *Oncorhynchus mykiss* in different farms from North Rhine-Westphalia, Germany, during the sampling period

Farm	Sampling times and water temperatures						Total
	June 2011 12–15°C	July 2011 15–18°C	Sept. 2011 10–15°C	Febr. 2012 4–5°C	April 2012 6–8°C	June 2012 8–12°C	
BR	81.8 (9/11)	62.5 (5/8)	37.5 (3/8)	41.7 (5/12)	33.3 (3/9)	0 (0/11)	42.4 (25/59)
NA			33.3 (2/6)				33.3 (2/6)
AL			100 (2/2)				100 (2/2)
FL	57.1 (8/14)	22.2 (2/9)	33.3 (2/6)			0 (0/3)	37.5 (12/32)
FR	0 (0/17)	0 (0/10)		0 (0/10)			0 (0/37)
SR	81.0 (17/21)	×[a]	33.3 (3/9)	50.0 (7/14)	56.3 (9/16)	62.5 (10/16)	60.5 (46/76)
ST			0 (0/3)		×[a]		0 (0/3)
KA	60.9 (14/23)	12.0 (3/25)	0 (0/6)	17.7 (3/17)		0 (0/21)[a]	21.7 (20/92)
PI	100 (12/12)	100 (4/4)	55.6 (5/9)	0 (0/4)	0 (0/5)	75 (9/12)	65.2 (30/46)
FI				0 (0/3)		60.0 (3/5)	37.5 (3/8)
MU		0 (0/8)			0 (0/8)	0 (0/5)	0 (0/21)
MS		0 (0/17)				0 (0/32)	0 (0/49)
Total	61.9 (60/97)	17.3 (14/81)	34.7 (17/49)	25.0 (15/60)	31.6 (12/38)	21.0 (22/102)	32.3 (139/431)

[a]Positive isolates were obtained before the sampling period

Table 3. Different genetic groups (TP: typing group) of *Yersinia ruckeri* present in rainbow trout *Oncorhynchus mykiss* farms in North Rhine-Westphalia, Germany

River system	Farm	Sampling times and water temperatures						Total
		June 2011 12–15°C	July 2011 15–18°C	Sept. 2011 10–15°C	Febr. 2012 4–5°C	April 2012 6–8°C	June 2012 8–12°C	
Lippe, Rhine	BR[a]	TP 17	TP 4	TP 20	TP 20	TP 20	–	TP 4, 17, 20
	NA			TP 21				TP 21
Lenne, Ruhr,	AL			TP 2, 20				TP 2, 20
Rhine	FL	TP 16	TP 16	TP 16			–	TP 16
	FR[b]	–	–		–			–
	SR[b]	TP 2, 19, 20	TP 3, 20	TP 2	TP 6	TP 2	TP 2, 8	TP 2, 3, 6, 8, 19, 20
Ruhr, Rhine	ST			–		TP 8		TP 8
Agger, Rhine	KA[a,b]	TP 2	TP 2	–	TP 7, 8	–	TP 26	TP 2, 7, 8, 26
	PI[a,b]	TP 6, 15	TP 2	TP 2, 22	–	–	TP 2	TP 2, 6, 15, 22
Rhine	FI[a]	–			–		TP 20	TP 20
Rur, Maas	MU		–			–	–	–
	MS		–				–	–
	Total	TP 2, 6, 15, 16, 17, 19, 20	TP 1, 2, 3, 4, 16, 20	TP 2, 5, 16, 20, 21, 22	TP 6, 7, 8, 20	TP 2, 8, 20	TP 2, 8, 20, 26	

[a]Farms vaccinating rainbow trout juveniles; [b]Farms with a trading relationship

TP were regularly found. In addition to isolates from TP 16, which were present during 3 sampling campaigns in farm FL, isolates from TP 2 were recovered regularly from trout in farms SR, KA and PI, and isolates from TP 20 were found regularly in farm BR. In farms BR, SR, KA and PI, additional isolates from further TPs were also present. Most of these isolates were only detected in a particular farm in trout from a single pond during 1 particular sampling campaign. Overall, isolates from 6 different TPs were present in farm SR and isolates from 4 TPs in farms KA and PI. *Y. ruckeri* from several TPs were recovered from the trout in June, July and September in farms SR and PI, while in farm KA the highest diversity of *Y. ruckeri* isolates was detected in February (Table 3). Hence, a seasonal pattern in the presence of particular TPs or in the presence of a higher diversity of *Y. ruckeri* isolates could not be observed (Table 3).

A specific array of isolates from particular TPs, which could be characteristic for a river system, could not be recognised in farms connected to the same river system including the Lenne, Ruhr or Agger Rivers (Table 3). In contrast to this, *Y. ruckeri* from specific TPs were present in farms KA, PI and SR, which had a trading relationship between each other. In all 3 infected farms, isolates from TP 2 were frequently detected, and isolates from TP 8 were present as well. Isolates from TP 20 were detected during every sampling campaign and in several farms (BR, AL, SR, PI) which had no obvious links (Table 3).

DISCUSSION

Since *Yersinia ruckeri*-induced ERM was reported for the first time in the USA (Rucker 1966), it has become an important infectious disease in trout hatcheries worldwide (Horne & Barnes 1999). The disease was initially controlled by vaccines prepared with classical motile *Y. ruckeri* strains (Johnson & Amend 1983), but after appearance and spreading of non-motile variants, the vaccine failed to control infections in Europe (Austin et al. 2003, Wheeler et al. 2009) and the USA (Arias et al. 2007). With the inclusion of both types of strains into the vaccine preparation, an enhancement of protection was achieved (Tinsley et al. 2011), but despite the use of those bivalent vaccines, ERM outbreaks occurred in vaccinated Atlantic salmon in Chile (Bastardo et al. 2011, Navas et al. 2014) as well as in European rainbow trout stocks. In 4 trout farms from the present study, the stocks were vaccinated with a bivalent vaccine, and in 3 of these farms, ERM outbreaks caused by non-motile strains were observed during several sampling campaigns. Infected juveniles or fry introduced into the farm for stocking might have served as a source of these infections (Tobback et al. 2007). The infections might also have originated from in-farm sources because the bacterium was observed to be able to survive in pond sediments or biofilms of tanks in trout farms (Coquet et al. 2002), which then could serve as a source for further disease outbreaks. In the current study, a molecular and phenotypic characterisation scheme for *Y. ruckeri* was applied

(Huang et al. 2013) to help trace the dissemination route of the *Y. ruckeri* isolates during recurrent disease outbreaks. This characterisation scheme revealed that in the majority of the farms, which were repeatedly sampled, bacteria from different TPs were found, and on several occasions, bacteria of different TPs were simultaneously present in the same farm. In most farms, however, ERM outbreaks were associated with the presence of *Y. ruckeri* from a distinct genetic group, which was characteristic for the specific farm. This could indicate that in those farms, ERM outbreaks most likely originated from sources such as latently infected fish stocks (Rodgers 1992) or contaminated environmental samples (Romalde et al. 1994, Coquet et al. 2002), which persisted in the farm rather than having been introduced from outside. The transmission of *Y. ruckeri* could also be related to wild fish, aquatic invertebrates (McDaniel 1971, Fuhrmann & Boehm 1983, Willumsen 1989) or birds (Bangert et al. 1988) as putative vectors. In the present study, we could not detect any indication for a contribution of these vectors to a dissemination of *Y. ruckeri* between the examined farms, as no *Y. ruckeri* strains from a common TP were detected in farms located in the same river system. In addition, farm FR, which had no previous history of *Y. ruckeri* infections and which was located on the same river together with ERM-positive farms, did not experience an ERM outbreak throughout the observation period.

In previous studies, mainly motile strains of *Y. ruckeri* were initially found associated with clinical infections of ERM in rainbow trout (Meier 1986). In the current study, isolates from most trout farms were non-motile, which might indicate that under the pressure of vaccination with vaccines directed against motile and non-motile *Y. ruckeri,* non-motile strains were distributed over several farms in NRW. In particular, bacteria from TPs 2, 8 and 20 were found most frequently. Bacteria from TPs 2 and 8 were isolated from 5 farms with a trading relationship between each other, which could facilitate the distribution of these particular types of the pathogen. However, bacteria from TP 20 were also found in several farms, which had no obvious trading relation.

Outbreaks of ERM were usually associated with challenging environmental conditions, such as poor water quality, excessive stocking densities and high water temperature (Horne & Barnes 1999). In this study, outbreaks were mainly observed in summer and early autumn, at a water temperature between 10 and 18°C. Especially, outbreaks caused by motile bacteria from TP 16 were observed during this period of time, supporting the hypothesis that motile strains are more active during warmer seasons (Huang et al. 2014). From June to July 2011, the rate of *Y. ruckeri*-positive rainbow trout decreased from 61.2 to 17.3%, as a result of the usage of antibiotic treatments. However, these measures could not provide 100% protection, and that may be why the positive rate increased again to 31.9% in September 2011. In some ponds, outbreaks of ERM were even observed during February and April, when the water temperature was below 10°C. These outbreaks were associated with non-motile *Y. ruckeri* strains, which were previously found to be more active at lower temperatures.

CONCLUSIONS

Mainly non-motile strains of *Yersinia ruckeri* could be isolated from vaccinated and non-vaccinated rainbow trout in NRW during the 13 mo sampling period. When *Y. ruckeri* isolates from different farms were characterised according to a detailed phenotypic and molecular characterisation scheme, in some farms *Y. ruckeri* from the same TP were recovered during ERM outbreaks, indicating that the infection originated from a source within the farm. In other farms, which had a trading relationship between each other, ERM outbreaks were caused by *Y. ruckeri* from the same TP, indicating a dissemination of the infection between the farms. The application of this characterisation scheme for pathogenic *Y. ruckeri* strains allows identifying sources of infections and routes of dissemination as a useful basis for disease prevention and monitoring plans.

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