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Yersinia ruckeri Biotypes 1 and 2 in France: presence and antibiotic susceptibility

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ABSTRACT: *Yersinia ruckeri* is the causative agent of yersiniosis, a disease reported in a number of fish species, especially rainbow trout. This study was undertaken to describe the phenotypes of *Y. ruckeri* on French rainbow trout farms. More than 100 isolates, collected during recent outbreaks on trout farms, were characterized by phenotypic tests, namely using biochemical tests of the API 20E system, serotyping, biotyping (tests for motility and lipase activity) and by describing the pattern of susceptibility to several antibiotics. The isolates showed a low phenotypic diversity with a prevalent serotype (O1) and API 20E profile 5 1(3)07 100. As in other European countries, Biotype 2 (BT2), which lacks both motility and secreted lipase activity, was found to be present in France. The emergence of 'French' BT2 was different than that observed for other European countries (Finland, Spain, Denmark and the UK). The antibiotic pattern was uniform for all isolates, regardless of the geographical area studied. The results indicate that no resistance has yet emerged, and the efficacy of the antibiotic generally used against yersiniosis in France, trimethoprim/sulfamethoxasol, is not compromised (minimum inhibitory concentrations [MIC] of between 0.016 and 0.128 µg ml^{-1}). Enrofloxacin and doxycycline, not used as a first-line treatment in fish diseases, have reasonably good efficacies (with MICs ≤0.128 and 0.256, respectively).

KEY WORDS: Yersiniosis · Serotype · Motility · Lipase activity · Resistance · Antimicrobial

INTRODUCTION

Yersinia ruckeri is the causative agent of enteric redmouth disease, also called yersiniosis. This agent mainly affects salmonid freshwater or seawater farms (genera *Oncorhynchus* and *Salmo*) (Austin & Austin 1993, Furones et al. 1993) but is also found in other fish families such as Ictaluridae (e.g. channel catfish; Danley et al. 1999). It was isolated and identified for the first time in 1956 on a rainbow trout farm in the Hagerman Valley, Idaho, in the USA, and this has come to be known as the Hagerman strain (Ross et al. 1966, Rucker 1966). In the 1980s, with the development of aquaculture and trade (fish and eggs), the bacterium was isolated in Europe (Stevenson et al. 1993), Australia (Bullock et al. 1978) and South Africa (Bragg & Henton 1986).

Y. ruckeri is a member of the *Enterobacteriaceae* family (Gram negative and oxidase negative). Various studies have established phenotypic characterizations of this pathogen using different methods. In 1984, according to a classification based on the whole cell antigen, *Y. ruckeri* was divided into 6 serovars: I, II, III, IV, V and VI (Stevenson & Airdrie 1984). Serovar I, with the ability to ferment sorbitol, represents Biotype 1 (BT1), while the other serovars (II to VI), which do not

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ferment sorbitol, correspond to Biotype 2 (BT2) (Stevenson & Airdrie 1984). Subsequent studies have, however, shown that the isolate corresponding to Serovar IV was not *Y. ruckeri* (De Grandis et al. 1988), thereby reducing the number of *Y. ruckeri* serovars to 5. Another approach to classification is based on the O-antigens (heat-stable antigens); this was first used to divide *Y. ruckeri* isolates into 5 serotypes (O1, O2, O5, O6 and O7) (Davies 1990), a later proposal reducing this 4 (O1, O2, O3 and O4) (Romalde et al. 1993), with subgroups within O1 and O2.

Studies of the virulence of *Y. ruckeri* have shown that Serotype O1 (Davies 1990), equivalent to Serovar I (Stevenson & Airdrie 1984), is predominantly isolated in clinical cases from rainbow trout farms (Wheeler et al. 2009, Haig et al. 2011). The motility and phospholipase activity of the serotype, rather than the ability to hydrolyse sorbitol as described by Stevenson & Airdrie (1984), were the 2 characteristics that allowed the serotype to be separated into 2 biotypes. BT1, also called the Hagerman biotype, was defined as motile and having phospholipase activity, in contrast to BT2, which does not have these characteristics (Davies & Frerichs 1989). For a long time, *Y. ruckeri* was considered phenotypically homogeneous (only BT1 having been observed) (Ewing et al. 1978, Stevenson & Airdrie 1984). However, with the development of aquaculture across the world and the emergence of BT2, it is now accepted that there is phenotypic variability mainly based on the presence or absence of flagella and, hence, motility.

Indeed, since 1989, the emergence and spread of BT2 in fisheries have been described (Davies & Frerichs 1989, Austin et al. 2003) and correlated with the recurrence of clinical reports and especially the failure of the commercial vaccine based on BT1. In several different countries — England (Austin et al. 2003), Spain (Fouz et al. 2006), the USA (Arias et al. 2007) and Finland (Ström-Bestor et al. 2010) — BT2 has been increasingly frequently isolated, associated with clinical cases. The emergence of BT2 seems to have multiple origins, the pattern varying considerably by geographical area and date of the analysis (Wheeler et al. 2009, Welch et al. 2011).

The present study, conducted between 2005 and 2009, was designed to (1) characterize the phenotypic variability of *Y. ruckeri* in France for the first time, (2) observe the presence of BT1 and/or BT2 as defined by Davies & Frerichs (1989), (3) compare the origins of BT2 (French or other origin), and (4) assess the pattern of susceptibility of *Y. ruckeri* to different antibiotics commonly used in veterinary and human medicine.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Isolates of *Yersinia ruckeri* were collected from different fish species in French fish farms in different counties, especially in the 2 most representative of the rainbow trout production: Brittany and Adour-Garonne. The majority of isolates was from disease outbreaks in rainbow trout farms over the period 2005 to 2009. They were collected from liver, spleen or kidney of fishes from 2 g to 2 kg in body mass. Several reference strains of *Y. ruckeri* were used: ATCC 29473 (Institut Pasteur, Paris, France), CECT 956 (University Valence, Spain), NCTC 12266, NCTC 12267, NCTC 12268, NCTC 12269, NCTC 12270 (Heath Protection Agency, London, UK) and TUTI EX5 (personal gift). For the determination of minimum inhibitory concentrations (MICs) and the antibiotic susceptibility tests, *Aeromonas salmonicida* ATCC 33658 (Institut Pasteur) and *Escherichia coli* ATCC 25922 (Institut Pasteur) were used as quality control strains.

All *Yersinia ruckeri* strains were grown on trypticase soy agar (Oxoid) at 22°C for 48 h. The same growth conditions were used for *Aeromonas salmonicida* ATCC 33658 and *Escherichia coli* ATCC 25922. Strains were stored at −80°C in trypticase soy broth (TSB) supplemented with 20% glycerol.

Identification of *Yersinia ruckeri*

For each isolate of *Y. ruckeri*, the identification was aided by classic phenotypical tests such as colony aspect, Gram coloration and oxidase tests (Merck). This phenotype identification was confirmed by standard PCR, using previously described primers (YER8 5′-GCG AGG AGG AAG GGT TAA GTG-′3 and YER10 5′-GAA GGC ACC AAG GCA TCT CTG-′3), with the production of a 575 bp amplicon (Gibello et al. 1999).

Phenotypic and biochemical tests

Several phenotypic and biochemical tests were performed to differentiate the isolates, in particular motility, lipase activity and API 20E profile.

Motility was determined by the microscopic observation of a bacterial suspension between a slide and cover slide and confirmed by a mannitol test using Mannitol Motility Nitrate (Bio Rad). Lipase activity

was tested by hydrolysis of Tween 80 on Schotts-Waltman agar at 22°C for 24 and 48 h. The API 20E profile was determined using an API 20E miniaturized system prepared according the manufacturer's protocol (Bio Mérieux) and incubating at 22°C for 48 h.

Genotyping of BT2

The genotyping of *Y. ruckeri* BT2 was performed as describe by Welch (2011). Briefly, *fliR*, *flhB* and *flhA* genes were amplified with fliRF+fliRR, flhBF+ flhBR and flhAF+flhAR primers, respectively. The amplicons were digested by specific restriction enzymes. The *fliR* gene was cut with *BstU*I and *Mnl*I to detect the *fliR*Δ1 and *fliR*Δ2 alleles, respectively, the *flhB* gene was cut with *Eae*I to detect the *flhB*Δ1 allele, and the *flhA* gene was cut with *Hpa*II to detect the *flhA* allele (Welch 2011). Products of PCR and digestion were observed on agarose gel (2%) after electrophoresis (100 V, 1 h). In parallel, all PCR products were sequenced (GATC Biotech) for observed mutations.

Antisera production and serotyping by slide agglutination

Antisera were obtained as described by Davies (1990), with some modifications. Briefly, the culture of each reference *Y. ruckeri* strain (Table 1) was inactivated by contact with formol for 24 h at room temperature. Heat-stable O antigens were obtained after heating bacterial suspensions at 98°C for 2 h. Cells were collected by centrifugation and resuspended in physiologic water plus formol.

For immunization, the cell concentration was adjusted to 2×10^9 CFU ml^{-1} in physiologic water. Rabbits were sacrified on Day 20 for antisera O1 and O2, on Day 25 for antisera O5 and O7, and on Day 32 for antiserum O6. The reactivity and specificity of each antiserum was tested with each reference strain by slide agglutination tests.

The antiserum O1 was absorbed with *Y. ruckeri* O5 to remove the non-specific agglutinins.

O-antigen serotyping of isolates was carried out by slide agglutination with the 5 specific rabbit antisera (O1, O2, O5, O6 and O7). Bacterial suspensions of an optical density (OD) of 1.8 at 580 nm were used. One drop of each isolate culture was mixed with one drop of each antiserum on glass slide. A distinct agglutination was registered as positive.

Determination of antibiotic susceptibility

Antibiotic susceptibility of each isolate was studied using 2 methods: the disc diffusion technique and MIC determination. *Aeromonas salmonicida* ATCC 33658 and *Escherichia coli* ATCC 25922 were used as the quality control strains as described by Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines.

Disc diffusion technique

Antibiotic susceptibility was determined by the disc diffusion technique on Mueller-Hinton agar (Biokar) in accordance with CLSI guidelines. A calibrate inoculum obtained by suspending 2 or 3 colonies in Mueller-Hinton broth (Oxoïd) and diluted in water with 0.9% NaCl to reach 10^6 CFU ml^{-1} was spread with a swab on a Mueller-Hinton agar plate and incubated at 22°C for 24 and 48 h, respectively, after disc deposition. The diameter of the inhibition halo was measured (in mm). The antimicrobial drugs tested and their concentration per disc were as follows: amoxicillin (25 µg), enrofloxacin (5 µg), erythromycin (15 µg), flumequine (30 µg), trime-

Table 1. Characteristics of *Yersinia ruckeri* reference strains used in this study. RT: rainbow trout. Asterisks indicate that the serotype was used for antisera

Reference strain no.	API 20E	Biotype	Serotype	Host	Location	Source
ATCC 29473	5 104 100	BT1	O1	RT	USA	Ewing et al. (1978)
CECT 956	5 305 500	BT1	O2	Fish	USA	Ewing et al. (1978)
NCTC 12266	5 304 100	BT1	O1*	RT	USA	Ewing et al. (1978)
NCTC 12267	5 305 500	BT1	O2*	Chinook salmon	USA	Ewing et al. (1978)
NCTC 12268	5 307 500	BT1	O5*	RT	UK	Ewing et al. (1978)
NCTC 12269	5 105 500	BT1	O6*	RT	Canada	Ewing et al. (1978)
NCTC 12270	5 304 500	BT1	O7*	Unknown	Denmark	Ewing et al. (1978)
TUTI EX5	5 107 100	BT2	O1	RT	UK	Austin et al. (2003)

thoprim/sulfamethoxasol (1.25/23.75 µg) (Bio-Rad), oxolinic acid (2 µg), gentamicin (10 µg), doxycycline (30 µg), oxytetracycline (30 µg) (Oxoïd) and florfenicol (30 µg) (Becton Dickinson).

MIC determination

For all strains, MICs were determined with the same antibiotics as in the disc diffusion technique. The MICs were determined with Sensititre plates (TREK Diagnostics Systems), which were inoculated with 50 µl of a 5×10^5 CFU ml^{-1} suspension in Mueller-Hinton broth (Oxoid). The antimicrobial concentrations (µg ml^{-1}) in the plate were as follows: enrofloxacin 0.004–2, amoxicillin 0.03–16, oxytetracycline 0.015–8, erythromycin 0.25–128, florfenicol 0.03–16, flumequine 0.015–8, doxycycline 0.03–16, oxolinic acid 0.015–4, gentamicin 0.06–4 and trimetoprim/sulfamethoxasol 0.015–1/0.3–19. The plates were incubated at 22°C under shaking for 24 h. MICs were defined as the lowest concentration of antibiotic that inhibited visible growth (turbidity).

Statistical analysis

To evaluate significant connections between phenotype (biotype, antimicrobial susceptibility) and physical parameters (sampling year, geographical origin), Kruskal-Wallis or Fisher tests were performed in the R environment (www.R-project.org/). A p-value of ≤0.05 was considered significant.

RESULTS

Identification and phenotyping of *Yersinia ruckeri* by biochemical tests

A total of 128 isolates of *Y. ruckeri* were studied from different fish species (124 rainbow trout, 3 sturgeon and 1 gudgeon), from a range of geographical regions in France: 55 isolates from 32 fish farms of Brittany, 53 from 33 fish farms of Adour-Garonne and 20 from 14 fish farms localized in other regions of France. Isolates were collected over a 4 yr period, from 2005 to 2009. Results by isolate are described in Table 2.

All isolates were Gram negative, oxidase negative and were positively identified by PCR using the primers Yer8–Yer10 (Gibello et al. 1999) (data not shown).

Of the 20 biochemical tests in the API 20E system, 4 gave results that varied between the *Y. ruckeri* isolates: utilisation of citrate, ability to produce a Voges-Proskauer (VP) reaction, ability to produce gelatinase (Gel) and ability to ferment sorbitol (Sor). On the basis of the results of these 4 tests, 10 API 20E profiles (described by 7-digit numerical codes) were obtained (5107100, 5307100, 5105100, 5305100, 5104100, 5304100, 5306100, 5107500, 5307500 and 5305500). Unfortunately, as it was sometimes difficult to interpret citrate reactions as positive or negative, this test could not be considered reliable for distinguishing between isolates. Hence, we assessed 6 API 20E profiles based on the combination of the 3 other tests (VP, Gel and Sor): 51(3)07100, 51(3)05100, 51(3)04100, 5306100, 51(3)07500 and 5305500 (Tables 2 & 3). The majority of the isolates (97 of 128) gave VP+, Gel+ and Sor– responses corresponding to the 51(3)07100 API 20E profile, and the second most dominant group (23/128) gave VP+, Gel– and Sor– responses corresponding to the 51(3)05100 API 20E profile. All isolates from rainbow trout (n = 124) were sorbitol negative, while for isolates from the other species (sturgeon and gudgeon) (n = 4), the sorbitol test was positive.

Based on motility and phospholipase activity, which characterise BT1 (isolates being motile and having phospholipase activity) and BT2 (isolates being non-motile with no detectable phospholipase activity) (Davies & Frerichs 1989), 74 isolates were identified as BT1 and 54 as BT2 (Table 3). Overall, the combination of the 3 specific API 20E tests (VP, Gel and Sor), along with the motility and phospholipase activity identified 9 phenotypes among the 128 isolates of *Y. ruckeri* (Table 3).

No mutation was observed by specific enzyme digestions (Welch 2011) or by sequencing of *fliR*, *flhB* and *flhA* genes (data not shown). Only the reference strain TUTI EX-5 from the UK presented mutation of *fliR* gene, as described by Welch (2011). The *fliR*Δ1 mutation was described, as involved in the BT2 origin of *Y. ruckeri* isolates from the UK or USA.

Serotyping of the *Yersinia ruckeri* isolates

The majority (n = 125) of the isolates were of Serotype O1. One isolate from rainbow trout was Serotype O5 and 2 from sturgeon were Serotype O6 (Tables 2 & 3). The isolate identified as O5 came from Adour Garonne and the 2 O6 isolates were from other unspecified regions. No cross reactions were observed for any of the isolates tested.

Table 2. List of isolates of *Yersinia ruckeri* used in the present study. AG: Adour-Garonne; B: Brittany; RT: rainbow trout

Strain no.	Origin	Host	API 20E	Biotype	Serotype
2005					
CAE 713	AG	Sturgeon	5305100	BT1	O1
CAE 433	Other	Sturgeon	5107500	BT1	O6
2006					
CAE 212	AG	RT	5107100	BT1	O1
CAE 220	AG	RT	5307100	BT1	O1
CAE 224	AG	RT	5107100	BT1	O1
CAE 222	AG	RT	5107100	BT1	O1
CAE 221	AG	RT	5107100	BT1	O1
CAE 223	AG	RT	5107100	BT1	O1
CAE 392	B	RT	5105100	BT1	O1
CAE 385	B	RT	5107100	BT1	O1
CAE 393	B	RT	5107100	BT2	O1
CAE 207	B	RT	5107100	BT1	O1
CAE 394	B	RT	5107100	BT1	O1
CAE 209	B	RT	5307100	BT2	O1
2007					
CAE 708	AG	RT	5307100	BT1	O1
CAE 706	AG	RT	5304100	BT2	O1
CAE 707	AG	RT	5307100	BT2	O1
CAE 711	AG	RT	5107100	BT2	O5
CAE 712	AG	RT	5107100	BT1	O1
CAE 709	AG	RT	5307100	BT1	O1
CAE 710	AG	RT	5304100	BT1	O1
CAE 396	B	RT	5304100	BT1	O1
CAE 397	B	RT	5305100	BT1	O1
CAE 399	B	RT	5107100	BT2	O1
CAE 398	B	RT	5107100	BT1	O1
CAE 400	B	RT	5307100	BT2	O1
CAE 705	B	RT	5306100	BT1	O1
CAE 431	Other	Sturgeon	5307500	BT1	O6
2008					
CAE 367	AG	RT	5107100	BT2	O1
CAE 427	AG	RT	5107100	BT1	O1
CAE 370	AG	RT	5305100	BT1	O1
CAE 429	AG	RT	5107100	BT2	O1
CAE 376	AG	RT	5107100	BT1	O1
CAE 374	AG	RT	5307100	BT1	O1
CAE 375	AG	RT	5307100	BT1	O1
CAE 648	AG	RT	5107100	BT1	O1
CAE 373	AG	RT	5107100	BT1	O1
CAE 361	AG	RT	5107100	BT2	O1
CAE 425	AG	RT	5107100	BT2	O1
CAE 428	AG	RT	5107100	BT2	O1
CAE 378	AG	RT	5107100	BT1	O1
CAE 368	AG	RT	5307100	BT2	O1
CAE 369	AG	RT	5307100	BT1	O1
CAE 363	AG	RT	5307100	BT1	O1
CAE 360	AG	RT	5307100	BT1	O1
CAE 380	AG	RT	5105100	BT2	O1
CAE 372	AG	RT	5307100	BT1	O1
CAE 358	AG	RT	5107100	BT1	O1
CAE 366	AG	RT	5107100	BT2	O1
CAE 359	AG	RT	5107100	BT2	O1
CAE 379	AG	RT	5307100	BT1	O1
CAE 405	B	RT	5105100	BT1	O1
CAE 408	B	RT	5105100	BT1	O1
CAE 407	B	RT	5107100	BT1	O1
CAE 654	B	RT	5307100	BT2	O1
CAE 658	B	RT	5107100	BT2	O1
CAE 410	B	RT	5107100	BT2	O1
CAE 409	B	RT	5105100	BT1	O1
CAE 413	B	RT	5105100	BT1	O1
CAE 653	B	RT	5107100	BT2	O1
CAE 404	B	RT	5105100	BT2	O1
CAE 420	B	RT	5105100	BT1	O1
CAE 415	B	RT	5105100	BT1	O1
CAE 414	B	RT	5104100	BT1	O1
CAE 656	B	RT	5305100	BT2	O1
CAE 655	B	RT	5305100	BT1	O1
CAE 403	B	RT	5107100	BT1	O1
CAE 406	B	RT	5107100	BT1	O1
CAE 417	B	RT	5105100	BT2	O1
CAE 650	B	RT	5307100	BT2	O1
CAE 657	B	RT	5307100	BT2	O1
CAE 418	B	RT	5107100	BT2	O1
CAE 419	B	RT	5105100	BT1	O1
CAE 652	B	RT	5307100	BT1	O1
CAE 412	B	RT	5105100	BT1	O1
CAE 651	Other	RT	5307100	BT2	O1
CAE 364	Other	RT	5107100	BT2	O1
CAE 365	Other	RT	5307100	BT1	O1
CAE 436	Other	RT	5107100	BT1	O1
CAE 649	Other	RT	5307100	BT2	O1
CAE 435	Other	RT	5307100	BT1	O1
CAE 362	Other	RT	5107100	BT2	O1
CAE 434	Other	RT	5307100	BT2	O1
CAE 432	Other	Gudgeon	5305500	BT1	O1
2009					
CAE 647	AG	RT	5307100	BT2	O1
CAE 641	AG	RT	5307100	BT2	O1
CAE 704	AG	RT	5307100	BT1	O1
CAE 702	AG	RT	5307100	BT1	O1
CAE 703	AG	RT	5107100	BT1	O1
CAE 454	AG	RT	5107100	BT1	O1
CAE 700	AG	RT	5307100	BT2	O1
CAE 699	AG	RT	5107100	BT1	O1
CAE 642	AG	RT	5307100	BT2	O1
CAE 644	AG	RT	5307100	BT2	O1
CAE 640	AG	RT	5107100	BT1	O1
CAE 645	AG	RT	5107100	BT1	O1
CAE 639	AG	RT	5107100	BT1	O1
CAE 643	AG	RT	5307100	BT1	O1
CAE 701	AG	RT	5307100	BT2	O1
CAE 646	AG	RT	5107100	BT1	O1
CAE 666	B	RT	5307100	BT2	O1
CAE 676	B	RT	5307100	BT2	O1
CAE 461	B	RT	5307100	BT1	O1
CAE 510	B	RT	5307100	BT2	O1
CAE 684	B	RT	5307100	BT1	O1
CAE 675	B	RT	5107100	BT2	O1
CAE 660	B	RT	5305100	BT1	O1
CAE 681	B	RT	5107100	BT2	O1
CAE 667	B	RT	5307100	BT2	O1
CAE 679	B	RT	5307100	BT2	O1
CAE 535	B	RT	5107100	BT2	O1
CAE 534	B	RT	5107100	BT2	O1
CAE 462	B	RT	5307100	BT2	O1
CAE 683	B	RT	5107100	BT1	O1
CAE 671	B	RT	5305100	BT1	O1
CAE 659	B	RT	5307100	BT2	O1
CAE 677	B	RT	5307100	BT1	O1
CAE 536	B	RT	5107100	BT1	O1
CAE 538	B	RT	5107100	BT2	O1
CAE 686	Other	RT	5305100	BT1	O1
CAE 668	Other	RT	5107100	BT2	O1
CAE 673	Other	RT	5105100	BT1	O1
CAE 669	Other	RT	5307100	BT2	O1
CAE 670	Other	RT	5307100	BT2	O1
CAE 680	Other	RT	5307100	BT2	O1
CAE 682	Other	RT	5307100	BT2	O1
CAE 678	Other	RT	5305100	BT1	O1
CAE 685	Other	RT	5105100	BT1	O1

Antibiotic susceptibility of the *Yersinia ruckeri* isolates

Isolate susceptibility to a range of antimicrobial agents was tested by the disc diffusion technique and MIC determination. The results of the 2 methods were correlated (data not shown). Given this, MIC results were used to describe the antibiotic susceptibility of the isolates.

Most isolates showed a uniform pattern of susceptibility for all of the antimicrobial agents tested. Results for 3 isolates (CAE 379, CAE 685 and CAE 710) were not determined. Statistical analysis showed no significant differences when comparing the sampling year or the biotype with the MIC distribution of all isolates (Kruskal-Wallis test, $p > 0.05$).

Only a few isolates presented resistance (MIC = 8 µg ml^{-1}) to florfenicol (n = 3) and flumequine (n = 1), while some others were highly sensitive to oxolinic acid (MIC ≤ 0.016 µg ml^{-1}; n = 5), flumequine (MIC = 0.032 µg ml^{-1}; n = 5) or enrofloxacin (MIC ≤ 0.008 µg ml^{-1}; n = 6; Table 4).

The most active antibiotic was trimethoprim/sulfamethoxasol, followed by enrofloxacin and doxycycline, with MICs <0.256 µg ml^{-1} for all isolates and median MICs of 0.032, 0.064 and 0.128 µg ml^{-1} for trimethoprim/sulfamethoxasol, enrofloxacin and doxycycline, respectively. Oxytetracycline, gentamicin, flumequine and oxolinic acid appeared to have moderate efficacy, with MICs ranging from 0.016 to 8 µg ml^{-1} with medians between 0.256 and 1 µg ml^{-1}. In contrast, both florfenicol and amoxicillin had poor activity, with MICs ≥0.256 µg ml^{-1} and medians between 1 and 2 µg ml^{-1} (Table 4). Finally, resistance to erythromycin was observed, with a median MIC of 8 µg ml^{-1}, an MIC range of 2 to 32 µg ml^{-1} and an inhibition diameter of 0 mm for some isolates (data not shown).

For 4 antibiotics (oxytetracycline, florfenicol, flumequine and oxolinic acid), the MICs for isolates from Adour-Garonne were significantly different (one dilution higher; Kruskal-Wallis test, $p < 0.05$) than those from the other regions (Table 4). A significant difference was observed for oxolinic acid between isolates from Brittany and those from other counties (Kruskal-Wallis test, $p < 0.05$). For oxytetracycline, flumequine and oxolinic acid, the MICs ranged from 0.256 to 1 µg ml^{-1} for the majority of isolates (n > 113). Most isolates (n = 94) were more sensitive to trimethoprim/sulfamethoxasol, with a relatively low MIC (0.032 µg ml^{-1}), with an MIC higher than 2 µg ml^{-1} for just one isolate. In contrast, florfenicol, which has a temporary authorization for use in French aquaculture, had high MICs (4 and 8 µg ml^{-1} for 14 and 3 isolates, respectively).

MICs for 2 antibiotics not used in aquaculture, enrofloxacin and doxycycline, were generally low, with similar responses for most isolates, 99 isolates having an MIC of 0.064 µg ml^{-1} for enrofloxacin and 91 isolates having an MIC of 0.128 µg ml^{-1} for doxycycline (Table 4).

DISCUSSION

Several studies have characterized *Yersinia ruckeri* isolates from various locations across the world or in specific countries (Wheeler et al. 2009, Bastardo et al. 2011, Tinsley et al. 2011). These characterizations are based on phenotypic analysis together with specific biochemical and serological tests and/or genetic

Table 3. Results for the analysis of biotype, API 20E-specific tests and serotypes for isolates of *Yersinia ruckeri*. Geographical zones and hosts are specified. Asterisks indicate species other than rainbow trout (RT) were used; the number of asterisks indicates the number of fish

Motility	Lipase activity	API 20E-specific test			No. of isolates by geographical zone				Host	Serotype
		VP reaction	Gel reaction	Sorbitol fermentation	France	Brittany	Adour-Garonne	Other		
BT1										
+	+	+	+	+	2	–	–	2**	Sturgeon	O6
+	+	+	–	+	1	–	–	1*	Gudgeon	O1
+	+	+	–	–	19	13	2*	4	RT, sturgeon	O1
+	+	+	+	–	48	13	32	3	RT	O1
+	+	–	–	–	3	2	1	–	RT	O1
+	+	–	+	–	1	1	–	–	RT	O1
BT2										
–	–	+	–	–	4	2	1	–	RT	O1
–	–	+	+	–	49	23	16	10	RT	O1, O5
–	–	–	–	–	1	1	1	–	RT	O1

Table 4. Minimum inhibitory concentration (MIC) of antimicrobial agents against *Yersinia ruckeri* isolates from France (values for Brittany, Adour-Garonne and other counties are specified). BT: biotype. **Bold**: isolates presumed to be highly susceptible; grey box: isolates presumed to be resistant. Asterisks indicate species other than rainbow trout were used; the number of asterisks indicates the number of fish

Antibiotic (family)	Geographical zone	BT	MIC (µg ml^{-1})														Median	Range
			0.004	0.008	0.016	0.032	0.064	0.128	0.256	0.512	1.000	2.000	4.000	8.000	16.000	32.000		
Oxytetracycline (cyclin)	Brittany	1							19	10							0.256	0.256–1.000
		2							17	8	1							
	Adour-Garonne	1							13*	15	5						0.512	0.256–1.000
		2							3	13	2							
	Other	1						1	6***	2							0.256	0.032–0.512
		2				**1**			6	3								
Flumequine (quinolone)	Brittany	1							1	21	7						0.512	0.256–1.000
		2								20	6							
	Adour-Garonne	1				**1***				10	17	3	1	1			1.000	0.032–8.000
		2								3	15							
	Other	1				**4*****				3	2						0.512	0.032–2.000
		2						1	1	5	2	1						
Oxolinic acid (quinolone)	Brittany	1							7	22							0.512	0.256–1.000
		2							13	12	1							
	Adour-Garonne	1			**1***					15	15	2					1.000	0.016–2.000
		2							1	8	9							
	Other	1		**1***	**3****				1	4							0.512	0.016–0.512
		2						1	3	6								
Trimethoprim/ sulfamethoxasol (sulfamide/ diaminopyridine)	Brittany	1		3	8	15	2	1									0.064	0.008–2.000
		2		2	3	20						1						
	Adour-Garonne	1		1	1*	30	1										0.032	0.008–0.064
		2				17	1											
	Other	1		1*	4**	4											0.032	0.008–0.032
		2		1	1	8												
Florfenicol (phenicol)	Brittany	1								2	21	6					1.000	0.512–2.000
		2								2	23	1						
	Adour-Garonne	1									3	18*	10	2			2.000	1.000–8.000
		2									3	10	4	1				
	Other	1							1		6**	2*					1.000	0.256–2.000
		2								1	8	1						
Gentamicin (aminoglycosid)	Brittany	1							5	17	7						0.512	0.256–1.000
		2							5	12	9							
	Adour-Garonne	1							10	16	7*						0.512	0.256–1.000
		2							5	12	1							
	Other	1							3	2	4***						0.512	0.256–2.000
		2							3	6		1						
Enrofloxacin (fluoroquinolone)	Brittany	1				1	25	3									0.064	0.032–0.126
		2				1	21	4										
	Adour-Garonne	1		**1***			29	3									0.064	0.008–0.126
		2					12	6										
	Other	1	**2**	**3*****			4										0.064	0.004–0.064
		2			1	1	8											
Amoxicillin (β-lactamin)	Brittany	1								1	22	6					1.000	0.512–4.000
		2								1	23	1	1					
	Adour-Garonne	1									27	3*	3				1.000	0.256–4.000
		2								1	14	3						
	Other	1								1	5*	1*	2*				1.000	0.256–4.000
		2							1		6	3						
Doxycycline (cyclin)	Brittany	1					2	22	5								0.128	0.064–0.256
		2					2	20	4									
	Adour-Garonne	1						25	8*								0.128	0.064–0.256
		2					1	10	7									
	Other	1					1	6*	2**								0.128	0.016–0.256
		2			**1**			8	1									
Erythromycin (macrolid)	Brittany	1										1		20	8		8.000	2.000–16.000
		2											1	12	13			
	Adour-Garonne	1										6	10	13	2	2*	8.000	2.000–32.000
		2											4	8	4	2		
	Other	1										1	1	3	1	3***	8.000	2.000–32.000
		2										1	1	4	4			

analysis with molecular techniques such as pulse field gel electrophoresis (PFGE) or PCR (Wheeler et al. 2009, Ström-Bestor et al. 2010, Bastardo et al. 2012b) or, more recently, multi-locus sequence typing (MLST) (Bastardo et al. 2012a). In our study, the variability observed in the biochemical and serological studies independently showed a high degree of homogeneity in the *Y. ruckeri* populations analysed.

Biotyping using the API 20E system identified 10 profiles for 128 isolates, the majority being 5107100 and 5307100 (differing in citrate utilisation), as observed in Finland (Ström-Bestor et al. 2010) and Spain (Fouz et al. 2006). Other numerical profiles elsewhere in the world have been described previously (Austin et al. 2003, Arias et al. 2007, Ström-Bestor et al. 2010). In our sampling, the ability to use sorbitol seems to be associated with the host species (Tables 2 & 3).

In serological studies, other authors have found a similar homogeneity of isolates, with most being the O1 serotype. The dominance of O1 is in accordance with the virulence associated with this serotype and in salmonid hosts (Haig et al. 2011). In the present study, a single isolate, which came from a rainbow trout farm in an Adour-Garonne isolate in 2007, was identified as the O5 serotype, as found in Atlantic salmon isolates from the UK in 1991 and from Norway in 1985. This is the first O5 rainbow trout isolate described in France. It will be interesting to continue studying the serotypes present, especially on this farm and in connected waters, to be able to conclude whether this is an isolated case or signals the emergence of this Serotype in France. Two other isolates were classified as Serotype O6. These both came from sturgeon and, even though Serotype O6 has been described in rainbow trout, it is possible that isolates of *Y. ruckeri* have a certain degree of host specificity.

For other phenotypic tests, especially those used for biotype characterization (motility and lipase activity), the majority of isolates in previous studies have been described as BT1 or BT2. Indeed, 97 isolates from Turkey presented similar responses for biochemical and phenotypic tests (VP test, gelatin hydrolysis, Tween 80 hydrolysis, motility and sorbitol fermentation) to the reference strain ATCC 29473 corresponding to BT1 (Onuk et al. 2011), as did the majority of isolates from Peru (n = 25/30) (Bastardo et al. 2011). However, in Finland (n = 23/25), the UK (n = 52/52) and Spain (n = 41/41), the most recent isolates have been classified as BT2 (Fouz et al. 2006, Wheeler et al. 2009, Ström-Bestor et al. 2010). In our study, the population of *Y. ruckeri* was more heterogeneous: both BT1 (58%) and BT2 (42%) were present, with an even geographical distribution (Fisher test, $p > 0.05$). Isolates were collected throughout the period 2005 to 2009 and the representation of BT2 was seen to increase from 2007 onwards across the country (Fisher test, $p = 0.047$). Specifically, before 2007, BT1 was clearly dominant but since then there has been a significant trend to a more even balance between the biotypes. In fact, the increase in BT2 in France has not just been steady but seems to be speeding up, suggesting that this biotype may be virulent. It will be interesting to characterise the biotype of more recent isolates of *Y. ruckeri* involved in outbreaks to validate this hypothesis. No mutation of *fliR*, *flhB* and *flhA* genes was the cause of emergence of BT2 in France. For French isolates, another, independent origin of emergence of BT2 than that observed for Finnish, Danish or Spanish isolates is supposed.

Unlike some other studies, we found no isolates that were non-motile but had lipase activity, a pattern that would indicate an absence of correlation between lack of motility and lipase activity (Tinsley et al. 2011). Indeed, this association (motility– and lipase+) seems to be associated with other salmonid species, such as *Onchorynchus mykiss*, *Salmo salar* and *Salvelinus alpinus*, and other species such as *Gadus morhua* (Tinsley et al. 2011).

Regarding antibiotic susceptibility, our results are consistent with studies on isolates from several other countries. MICs were mostly similar for oxolinic acid, gentamicin, trimethoprim, enrofloxacin and oxytetracycline (De Grandis & Stevenson 1985, Onuk et al. 2011), with the exception being that some isolates had high MICs for oxytetracycline, ranging between 1 and 256 µg ml^{-1} (De Grandis & Stevenson 1985). Generally, *Y. ruckeri* is sensitive to amoxicillin, enrofloxacin, oxytetracycline, trimethoprim, ampicillin and flumequine, while it is resistant to erythromycin (De Grandis & Stevenson 1985, Akhlaghi & Sharifi Yazdi 2008, Onuk et al. 2011). Our results are in accord with the natural sensitivity observed for all antibiotics used in our study, except erythromycin, for which a natural resistance was described for the *Yersinia* genus (Stock et al. 2002). In our study, amoxicillin had poor efficacy, with MICs of 0.256 to 4 µg ml^{-1}, as did florfenicol, with MICs of 0.256 to 8 µg ml^{-1}. Similar results were obtained for Danish isolates, with MICs of 4 to 16 µg ml^{-1} for amoxicillin and 4 to 8 µg ml^{-1} for florfenicol (Schmidt et al. 2000). Overall, the MIC distributions for these antibiotics were homogeneous, and this makes it difficult to distinguish evolution of resistance in the population. As can be seen in Table 4, some isolates presented a 'break point', with a high susceptibility or a supposed resistance to some antibiotics. For flumequine and oxolinic acid, only sturgeon and gudgeon isolates presented a high susceptibility compared with rainbow trout isolates. The susceptibility could be explained by a lower level of exposure to these antibiotics for these fish species than is the case in the intensive production of rainbow trout. For enrofloxacin, the same phenomenon is observed, with a

high susceptibility for isolates from other fish species and for one isolate of rainbow trout from counties other than the 2 major production counties (Brittany and Adour-Garonne). In the present study the susceptibility of sturgeon and gudgeon isolates to antibiotics used in French aquaculture and to enrofloxacin was higher than that of rainbow trout isolates (Kruskal-Wallis test, $p < 0.05$). As with other phenotypic characteristics (sorbitol use), the antibiotic susceptibility of isolates could be associated with the fish host species. In this study, supposed resistance was observed only for 6 isolates: 2 for flumequine (4 and 8 µg ml^{-1}), 1 for trimethoprim/sulfamethoxasol (2 µg ml^{-1}) and 3 for florfenicol (8 µg ml^{-1}; Table 4). Except for the resistance observed for trimetoprim/sulfamethoxasol, for which the isolate came from Brittany, the other isolates were from Adour-Garonne. In our study, we found sensitivities to trimethoprim/sulfamethoxasol and enrofloxacin higher than those reported for isolates from Iran, Peru and Turkey (Akhlaghi & Sharifi Yazdi 2008, Bastardo et al. 2011, Onuk et al. 2011). The use of enrofloxacin could be efficient in aquaculture, but in France it has been restricted to use in veterinary medicine since 1991, due to the risk of the development of bacterial resistance.

Interestingly. a similar response is seen across countries with different practices. Even higher MICs were observed for oxolinic acid in a previous Danish study (Schmidt et al. 2000). Denmark has been a producer of salmonids for many years, which could explain the change in the susceptibility of the pathogen. Currently, new countries are emerging as producers. For example, Iran has become an important producer of rainbow trout; in studies to date, no resistant isolates have been detected here, but it is important to carefully monitor possible emergence.

CONCLUSION

In conclusion, the French strains of *Yersinia ruckeri* collected from trout farms between 2005 and 2009 have biochemical and serological characteristics that are very homogeneous. The antibiotic susceptibility was homogenous for our national sampling. However, this study revealed the presence of BT2 as well as BT1 in French trout farms. It will be interesting to monitor the evolution of the ratio between the biotypes, and differentiate isolates of *Y. ruckeri* by biotype for use in the treatment and control of yersioniosis.

A suitable focus for future study would be to explore the genetic field of these strains, using techniques such as PFGE and/or MLST. In particular, it would be interesting to ascertain whether genetic analysis reveals a greater diversity than the phenotypic study and, if so, if this could help our understanding of the emergence of BT2 in France. This genetic approach could provide data valuable for vaccine development.

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