

Antimicrobial resistance of *Yersinia enterocolitica* and presence of plasmid pYV virulence genes in human and animal isolates

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Abstract

Interactions between bacterial virulence and antimicrobial resistance are of increasing interest in clinical microbiology. On this account, antimicrobial resistance of *Yersinia enterocolitica* O:3 strains isolated from humans ($n = 55$), food-chain animals ($n = 58$) and companion animals ($n = 13$) was determined in relation to the absence or presence of the pYV plasmid-encoded virulence genes *yadA* and *virF*. There were no statistically significant associations between the rate of antimicrobial resistance and the presence or absence of the plasmid, in either human-derived or animal-derived strains. Therefore, it can be concluded that response to conventionally used antimicrobials in *Y. enterocolitica* O:3 strains is not dependent on pYV-encoded virulence determinants.

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Introduction

Yersinia enterocolitica is a member of the family *Enterobacteriaceae* and a widely distributed gastrointestinal pathogen transmitted via the faecal–oral route after consumption of contaminated food or contact with infected or colonized animals. Although the majority of infections are self-limiting, antimicrobial therapy is warranted to treat immunocompromised individuals and patients with sepsis or invasive infections. Most pathogenic *Y. enterocolitica* strains associated with human disease belong to serogroups O:3, O:9, O:8, O:5,27 and express both chromosome- and plasmid-encoded virulence determinants [1].

Enteropathogenicity of *Y. enterocolitica* is conditional on the presence of the highly conserved virulence plasmid pYV (approximately 70 kb), which contains among others the gene *yadA* and its transcriptional activator *virF*. The gene *yadA* encodes a protein essential for induction of disease by *Y. enterocolitica*, as it subserves key virulence functions of the pathogen, such as adhesion and serum resistance [2]. The gene *virF* encodes a DNA-binding protein that regulates positively the transcription of *yadA* as well as of other plasmid-encoded outer membrane proteins [3].

Accumulating evidence suggests that there are multiple interactions between antimicrobial resistance and bacterial virulence, possibly promoting the selection of high-risk clones (reviewed in [4–6]). Given that such interactions are not known for *Y. enterocolitica*, we set out to explore an association on a statistical level. The pYV plasmid plays a critical role in guiding the invasion and survival of virulent *Yersinia* strains in the human host [7], hence we hypothesized that the absence or presence of the plasmid might be associated with the rates of antimicrobial resistance in *Y. enterocolitica* isolates. As the pathogen is transmitted through consumption of contaminated food or contact with

animals, we also examined potential associations in strains derived from food-chain and companion animals.

Materials and methods

Sample collection

A total of 835 asymptomatic food-chain animals at slaughter (pigs, sheep, chickens and cows) were sampled during the period 1999–2002 from several geographical departments of Greece, as previously described [8]. Eighty-three food-chain animals were found positive for *Yersinia* spp., 76 of which belonged to *Y. enterocolitica* and were used in the present study.

Additionally, 21 *Y. enterocolitica* isolates from symptomatic companion animals (dogs and cats) from France and Italy were used, which were kindly provided by Dr Peter Kopp (Vet Med Labor GmbH, IDEXX Laboratories, Ludwigsburg, Germany) to the Friedrich-Loeffler Institut in Jena, Germany.

Finally, 55 *Y. enterocolitica* strains isolated between 2006 and 2011 from stool and blood cultures from hospitalized children with β -thalassaemia with gastroenteritis [9] were used for the comparison of strains found in animals and humans.

Serotyping

Serotyping was performed by slide agglutination with commercially available specific O:3 and O:9 anti-sera (Sifin Diagnostics, Berlin, Germany). All human-derived strains belonged to O:3, so no other anti-sera were used for further subtyping (see also Results).

DNA isolation

DNA was isolated from pure bacterial cultures using the High Pure PCR Template Preparation kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Detection of plasmid-encoded virulence genes

For the amplification of *yadA* and *virF/lcrF* fragments, a PCR protocol based on Thoerner et al. [10] was used. *YadA* was detected using the primers *yadA* forward (5'-CTTCAGATACTGGTGTGCTGT-3') and *yadA* reverse (5'-ATGCCTGACTAGAGCGATATCC-3') specific for an 849-bp amplicon and *virF/lcrF* was detected using *virF/lcrF* forward (5'-GGCA-GAACAGCAGTCAGACATA-3') and *virF/lcrF* reverse (5'-GGTGAGCATAGAGAATACGTCG-3') specific for a 561-bp amplicon (Jena Bioscience, Jena, Germany).

Antimicrobial susceptibility testing

Determination of antimicrobial susceptibility was performed according to the CLSI/NCCLS criteria [11] using a standardized microdilution susceptibility test (MICRONAUT-S; Merlin

Diagnostika, Bornheim, Germany). Nine antimicrobial agents were chosen based on conventional clinical therapeutic implementation: ampicillin, amoxicillin/clavulanic acid, ceftazidime, cephalothin, ciprofloxacin, co-trimoxazole, erythromycin, gentamicin and tetracycline.

Statistical analysis

One-way analysis of variance (ANOVA) was used for statistical analysis. For parametric testing, one-way ANOVA was used to detect the significant effects of variables to compare the means across groups. The differences of the means were considered significant if $p < 0.05$ and the F value (variation between sample means/variation within the samples) was greater than F critical.

Results

Fifty-eight *Y. enterocolitica* strains deriving from food-chain animals were of serotype O:3, and 18 strains belonged neither to O:3 nor to O:9 serotypes. All O:3 strains in this group derived from pigs. Regarding companion animals, 13 *Y. enterocolitica* strains belonged to serotype O:3, 6 strains were O:9 and 2 strains were non O:3, non O:9. By contrast, all 55 human isolates belonged to serotype O:3. Therefore, to improve homogeneity, we restricted the comparison of strains across species to this serotype (Tables 1 and 2).

We first calculated the percentages of human- and animal-derived strains showing resistance to antimicrobial agents irrespective of the presence or absence of the pYV plasmid (Table 1). ANOVA among the three groups showed no statistically significant difference ($F = 0.76$, F critical = 3.4, $p = 0.47$), suggesting that antimicrobial resistance rates are not different overall among *Y. enterocolitica* strains derived from humans, food-chain animals and companion animals.

Next, we calculated the percentages of resistant strains within each group in relation to the presence ($yadA^+ virF^+$) or absence ($yadA^- virF^-$) of the pYV plasmid (Table 2). ANOVA among the three groups showed that the presence or absence of the plasmid does not have a significant effect on the overall resistance rates of the isolated strains ($F = 0.19$, F critical = 4.02, $p = 0.66$). Similarly, within-groups ANOVA analyses yielded no significant results (all values $p > 0.40$) and all F values were lower than the respective F critical. There were also no significant differences after excluding early-generation β -lactam antibiotics (ampicillin, cephalothin) and β -lactam/inhibitor combinations (amoxicillin/clavulanic acid), to which *Y. enterocolitica* exhibits intrinsic resistance ($F = 0.02$, F critical = 4.13, $p = 0.95$). Hence, we conclude that there is no association between the pYV plasmid and antimicrobial resistance, either across groups or individually within each group of the *Y. enterocolitica* strains herein tested.

TABLE 1. Antimicrobial resistance rates of *Yersinia enterocolitica* O:3 strains isolated from humans, food-chain animals and companion animals

| Antimicrobial | No. of resistant strains | | |
|------------------|--------------------------|-----------------------------|----------------------------|
| | Human (n = 55) | Food-chain animals (n = 58) | Companion animals (n = 13) |
| Amoxicillin/clav | 0 | 9 (15.52) | 0 |
| Ampicillin | 43 (78.18) | 57 (98.27) | 2 (15.38) |
| Ceftazidime | 0 | 6 (10.34) | 0 |
| Cephalothin | 54 (98.18) | 58 (100) | 12 (92.31) |
| Ciprofloxacin | 0 | 0 | 0 |
| Co-trimoxazole | 0 | 3 (5.17) | 0 |
| Erythromycin | 55 (100) | 58 (100) | 5 (38.46) |
| Gentamicin | 0 | 16 (27.59) | 0 |
| Tetracycline | 0 | 7 (12.07) | 1 (7.69) |

Values in brackets refer to % of the total of each group.

TABLE 2. Antimicrobial resistance in relation to the absence or presence of plasmid-encoded virulence genes in *Yersinia enterocolitica* O:3 strains isolated from humans, food-chain animals and companion animals

| Antimicrobial | Total resistant | Plasmid-encoded virulence genes | |
|-----------------------------|-----------------|-------------------------------------|-------------------------------------|
| | | yadA ⁻ virF ⁻ | yadA ⁺ virF ⁺ |
| Human | | | |
| Amoxicillin/clavulanic acid | 0 | — | — |
| Ampicillin | 43 | 35 (81.40) | 8 (18.60) |
| Ceftazidime | 0 | — | — |
| Cephalothin | 54 | 35 (64.81) | 19 (35.19) |
| Ciprofloxacin | 0 | — | — |
| Co-trimoxazole | 0 | — | — |
| Erythromycin | 55 | 36 (65.45) | 19 (34.55) |
| Gentamicin | 0 | — | — |
| Tetracycline | 0 | — | — |
| Food-chain animals | | | |
| Amoxicillin/clavulanic acid | 9 | 2 (22.22) | 7 (77.78) |
| Ampicillin | 57 | 16 (28.07) | 41 (71.93) |
| Ceftazidime | 6 | 1 (16.67) | 5 (83.33) |
| Cephalothin | 58 | 17 (29.31) | 41 (70.69) |
| Ciprofloxacin | 0 | — | — |
| Co-trimoxazole | 3 | 0 | 3 (100) |
| Erythromycin | 58 | 17 (29.31) | 41 (70.69) |
| Gentamicin | 16 | 2 (12.5) | 14 (87.5) |
| Tetracycline | 7 | 0 | 7 (100) |
| Companion animals | | | |
| Amoxicillin/clavulanic acid | 0 | — | — |
| Ampicillin | 2 | 1 (50) | 1 (50) |
| Ceftazidime | 0 | — | — |
| Cephalothin | 12 | 1 (8.33) | 11 (91.67) |
| Ciprofloxacin | 0 | — | — |
| Co-trimoxazole | 0 | — | — |
| Erythromycin | 5 | 1 (20) | 4 (80) |
| Gentamicin | 0 | — | — |
| Tetracycline | 1 | 0 | 1 (100) |

Values in brackets refer to % of the total of each group.

increasingly observed in several bacterial species, including *Enterobacteriaceae* [5,6], we hypothesized that the presence of *yadA*, along with its transcriptional activator *virF*, would be associated with antimicrobial resistance in virulent strains of *Y. enterocolitica* isolated from humans and animals. Nevertheless, we failed to detect such an association, in either human- or animal-derived strains.

It is well known that various strains of *Y. enterocolitica* exhibit resistance to β -lactam antibiotics, such as ampicillin and cephalothin, with the degree and spectrum of resistance depending on the differential expression and activity of two distinct, chromosomally encoded β -lactamases [14–17]. To rule out potential effects of intrinsic resistance to early generation β -lactams and β -lactam/ β -lactamase inhibitor combinations, we repeated our analyses following exclusion of ampicillin, cephalothin and amoxicillin/clavulanic acid. This did not essentially change our results.

Certain limitations of our study should be discussed. First, our approach was merely correlational, so our analyses could not have yielded results equivalent to experimental work. Our rationale was to zoom out and explore if an association between the plasmid and antimicrobial resistance exists on a statistical level. Had we found an association, it would then justify subsequent plasmid analysis experiments to identify the exact genetic elements involved. Second, data on previous antimicrobial exposure were not available, because this information cannot be safely reported in a retrospective manner. In addition, no relevant information was disclosed by slaughterhouses about food-chain animals and rarely did veterinarians report such information about companion animals. Third, no associations could be examined between the plasmid and clinical parameters, such as disease severity or outcome, as the groups are characterized by different clinical presentations and host reactions to the pathogen. Namely, humans and companion animals were reportedly symptomatic, whereas food-chain animals were asymptomatic and merely colonized.

Discussion

Encoding the predominant virulence factor *YadA*, the plasmid pYV constitutes a crucial virulence marker of *Y. enterocolitica* [1, 12]. *YadA* acts both as an adhesin and as an invasin [2], and in O:3 strains it is indispensable for long-term serum resistance [13]. As co-selection of virulence and resistance determinants is

Lastly, as *Y. enterocolitica* is not a frequent enteropathogen, collection of strains from symptomatic humans and companion animals could only be accomplished prospectively. In contrast, strains from colonized—but otherwise healthy—food-chain animals were collected upon scheduled appointments with the slaughterhouses. Hence, by definition, sampling periods were different across groups. This difference, however, is conceivably of minimal relevance to our results, because any association between antimicrobial resistance and the presence of the plasmid-encoded genes herein tested is not expected to have changed in the short interval between the sampling periods.

We conclude that no association exists between the plasmid pYV and resistance to conventionally used antimicrobials. Although plasmids encoding both resistance and virulence factors have been described in other *Enterobacteriaceae* [5], simultaneous selection of resistance factors and virulence determinants is less likely to have occurred in the pYV plasmid. This however does not rule out the possibility of existence of other plasmids that could have served as genetic carriers of both resistance- and virulence-associated genes.

Conflict of interest

The authors declare no conflict of interest.

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