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► **To cite this version:**

Michel C, Faivre Bernadette. IN VITRO AND IN VIVO STUDY OF AN ANTIMICROBIAL ACTIVITY DISPLAYED BY THE REDMOUTH DISEASE AGENT, YERSINIA RUCKERI. *Annales de Recherches Vétérinaires*, 1987, 18 (1), pp.43-46. hal-00901688

HAL Id: hal-00901688

<https://hal.science/hal-00901688v1>

Submitted on 11 May 2020

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IN VITRO AND IN VIVO STUDY OF AN ANTIMICROBIAL ACTIVITY DISPLAYED BY THE REDMOUTH DISEASE AGENT, *YERSINIA RUCKERI*

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Received, 10th May, 1986/Accepted, 12th June 1986

Résumé

ÉTUDE *IN VITRO* ET *IN VIVO* D'UNE ACTIVITÉ ANTIMICROBIENNE MANIFESTÉE PAR *YERSINIA RUCKERI*, AGENT DU «RED MOUTH DISEASE». — Un facteur antimicrobien soluble a été détecté chez *Yersinia ruckeri* cultivée en milieux solides. Les techniques de contact et de double couche ont permis d'en préciser la nature bactériostatique, et de noter la particulière sensibilité d'autres bactéries pathogènes pour les poissons (*Aeromonas*, *Vibrio*). Par contre les autres souches de *Y ruckeri* étaient résistantes. On n'a trouvé aucune corrélation entre cette activité antimicrobienne et la virulence des souches, et des tentatives d'infections expérimentales simultanées par la yersiniose et la furunculose n'ont pas empêché l'évolution d'infections mixtes. La nature et le rôle épidémiologique de ce facteur sont encore à préciser.

First reports of redmouth disease epizootics in Europe arised simultaneously in 1981 (Fuhrmann *et al* 1983, Lesel *et al* 1983, Roberts 1983). Whatever the exact origin of the disease, the striking fact has been its fast and unforeseen spread. In few years it became one of the main plagues in salmonid culture and, at the same time that it progressed, usual complaints of professionals about other bacterial diseases, like furunculosis or vibriosis tended to diminish (Ghittino, personal communication). One could wonder if such a feeling was just of psychologic nature, or if some objective properties of the causative agent, *Yersinia ruckeri*, might account for it. This paper affords some evidence of an antimicrobial factor secretion by *Y ruckeri*. As it was not possible to detail in a short note all our attempts to characterize the substance, which will be reported later, only biological aspects and possible role in epidemiology of yersiniosis will be discussed.

Materials and Methods

In vitro studies were performed on glass Petri dishes according to two classic techniques. A contact droplets technique (Rosebury *et al* 1954) allowed to assess the susceptibility of different bacterial species to the product. Droplets of *Y ruckeri* broth cultures were laid down on tryptic-soya agar (TSA) and incubated 24 h at 22 °C, after what bacteria were killed by chloroform vapors, 30 min at 37 °C. Droplets of diluted sensitive cultures (dilutions ranged between 1/20 and 1/200 depending on the species) were then added in such

manner that they overlapped the edge of the inactivated bacterial spots. Inhibition pictures could be seen after 24 or 48 h incubation at 22 °C (fig 1). Five strains of *Y ruckeri*, isolated in France from clinical cases, were tested against sets of three or five strains of the species listed in table 1. Three strains of *Y ruckeri* were also tested in cross reactions.

The double agar layer method of Fredericq and Levine (Hamon 1985) proved better to get a quantitative evaluation of the product released in solid media. *Yersinia* cultures were prepared as above, but the susceptible strain broth was mixed at 1/100 in 5 ml of agar 7 % previously melted and held at 45 °C. The agar was poured out onto the killed *Yersinia*, and after incubation, diameters of inhibition areas could be measured and compared. This method permitted to optimize the media and the cultural conditions in order to obtain clearer and larger inhibition pictures. So a meat peptone (Réf 19521, Organotechnie, 93120 La Courneuve, France) adjusted at pH 6.2 with phosphate buffer provided the best results when producing strains were incubated 48 h at 22 °C. It was then possible to punch out agar cylindrical samples inside the inhibition area, and to transfer them into nutrient-broth tubes for detection of surviving bacteria.

In vivo experimentation aimed to investigate what happened in case of a double infection involving *Y ruckeri* and the causative organism of furunculosis, *Aeromonas salmonicida*. Fingerlings of rainbow trout (*Salmo gairdneri*) bred in the laboratory facilities and weighing about 8-10 g were distributed in 12 l aquaria supplied with flowing-through dechlorinated tap water heated at 14 °C. Each aquarium received 15 fingerlings. Bacterial strains *Y ruckeri* TG 38/84 and *A salmonicida* TG 72/78 were selected for their virulence and grown in stirred Erlenmeyer flasks containing 200 ml of TSB,

according to our standard procedure (Michel 1980). Twenty-four hours cultures were harvested, centrifugated for 25 min at 2 000 g and pellets were resuspended in tap water in order to make up pre-dosed samples containing about 2×10^{10} bacteria/ml. When added to aquaria in which the water level had been adjusted at 2 l, these doses allowed to perform bath infections of one hour in 10^7 bacteria/ml. Three aquaria received *Y ruckeri*, three other ones *A salmonicida*, and the last three were simultaneously submitted to mixed infections. Doses were checked by the droplets method (Miles and Misra 1938). Cumulative mortalities were recorded, and kidneys from both moribund and dead fish were sampled on TSA for identification of colonies (easily recognizable through 45° transmitted light examination).

It was possible to detect a variant strain of *Y ruckeri*, TG 24/84, defective for the production of the antimicrobial factor. Virulence of this strain and of an original one, TG 67/83 was compared in trout of the same origin as above. Tenfold dilutions of bacterial cultures were injected intraperitoneally in groups of 10 fish held at 14 °C. Mortality curves were plotted for comparison.

Results and discussion

Some examples of pictures observed with the contact droplets technique are shown in the figure 1. From absence of effect to complete inhibition of growth, three degrees of positive reactions could be roughly considered, and permitted to compare the susceptibility of different bacterial strains as shown in table 1. It clearly appeared

that Gram negative germs, namely those of the *Vibrionaceae* family, were the most sensitive to the inhibiting factor. Gram positive bacteria and *Pseudomonas* species proved resistant, and no evidence of self inhibition or intra-specific competition could be found. All the agar samples taken off in the inhibition areas of double agar layers and seeded in nutrient broth were able to revive after a variable lag time. Although its real nature remains unknown for the moment, the inhibitory factor seems to be bacteriostatic, and does not inhibit *Y ruckeri* strains, that is not in accordance with the properties of a bacteriocin. It must be noted that its production in liquid media is trifling or very difficult to detect, what renders further studies questionable.

Trout infected by immersion route proved very susceptible to experimental diseases. This was not a surprise with *Y ruckeri*, since the control numeration confirmed an infective dose of 5×10^7 bacteria/ml. However the dilution procedure was not so accurate with *A salmonicida*, and only 2×10^5 bacteria/ml were administered. So, the experimental furunculosis was milder than expected. After 15 days mortalities were respectively 86.5 % for redmouth controls, 30 % for furunculosis controls, and 95.5 % for doubly infected fish. Thirty-six dead or dying fishes of the last group were kept for bacteriological examination. In 12 of them (33.3 %), *Y ruckeri* was identified in pure

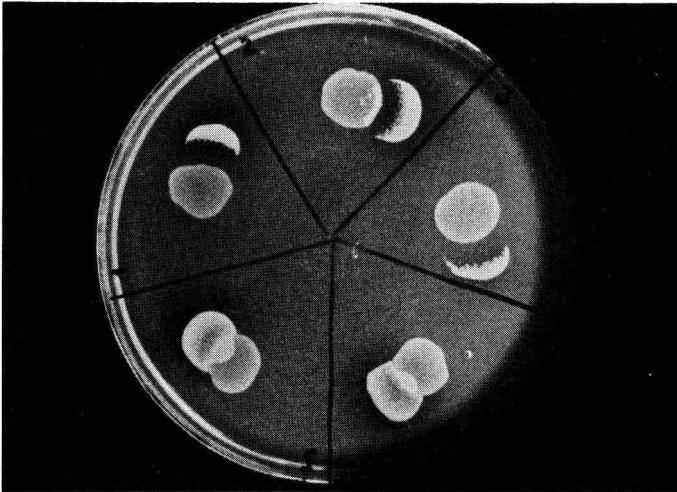


Fig. 1. — Inhibiting effect of five *Y ruckeri* strains visualized against a single *A hydrophila* strain using the contact droplets technique. Reactions 1 to 3 were moderately positive (2, in our reading scale, cf table 1), 4 and 5 unefective (0).

culture. The remaining 24 fishes (66.6 %) hosted both *Yruckeri* and *A salmonicida*. It could be concluded that yersiniosis infection does not prevent furunculosis from developing in fish organisms. This is in accordance with unpublished observations recently reported by HD Rodger, CJ Rodgers and G Giorgetti (2nd international conference of the EAFF, 2-5 September 1985, Montpellier). These authors noticed some cases of mixed infections with *Yruckeri* and *A salmonicida*, and even *Yruckeri* and *Vibrio anguillarum*. In fact all these pathogens can penetrate and colonize different sites of the fish body and there was no theoretical reason to deny the possibility of simultaneous infections. Moreover, the inhibiting factor of *Yruckeri* is not equally released in all kinds of media, and it is not sure that fish tissues provide optimal conditions for its production. It is likely that if *Yruckeri* had really some advantage on other bacteria, the mechanisms of this competition should be investigated elsewhere than in fish.

A last point of interest was the checking of possible relationships between the pathogenicity of the strains and their ability to produce the

bacteriostatic factor. Parenteral injections of producing and non-producing strains dit not allow to compute LD50 as for other pathogens. As in previous experimentations it appeared that when trout are highly susceptible to yersiniosis the recorded mortalities are the same whatever the dose of infection. This can be seen in the table 2, which also reveals a similar virulence of the two strains. The production of the inhibiting factor cannot serve as a marker for the virulence.

To sum up, an antimicrobial product excretion has been demonstrated in *Yruckeri* cultures. This product is bacteriostatic, and although it does not seem to fit with bacteriocins characteristics; its real nature has still to be determined. It is not associated to the virulence, and it does not appear to be released or confer any advantage over other bacterial fish pathogens under clinical conditions. Of course, it may be merely a metabolic waste without practical interest, but the special sensitivity of *Vibrionaceae* family members, in which many important fish pathogens are listed, together with field observations, should prompt to undertake further investigations.

Table 1. - Comparative sensitivity of different bacterial species to the antimicrobial factor produced by *Yruckeri* (percentages of observations are related to the number of strains combinations involving *Yruckeri* and the tested species).

Tested species	Number of strains combinations	Degrees of inhibition (a)			
		0	1	2	3
<i>Yersinia ruckeri</i>	9	100	0	0	0
<i>Vibrio anguillarum</i>	15	0	0	0	100
<i>Aeromonas hydrophila</i>	25	36	12	28	24
<i>Aeromonas salmonicida</i>	25	4	24	12	60
<i>Pseudomonas fluorescens</i>	25	88	12	0	0
<i>Flexibacter</i> spp	25	36	60	4	0
<i>Lactobacillus piscicola</i>	15	100	0	0	0

(a) 0 : none ; 1 : inhibition in overlapping area ; 2 : clear crescent of culture ; 3 : slight crescent, or no culture

Table 2. - Comparison of mortalities among 10 g rainbow trout fingerlings, 15 days after intraperitoneal injection of increasing doses of *Yruckeri* antimicrobial factor producing strain TG 67/83 and non producing strain 24/84

<i>Yruckeri</i> inoculated strain	dose: (log ₁₀ no. bacteria) ^a					
	1.78	2.78	3.78	4.78	5.78	6.78
	(% dead fish)					
TG 67/83	90	60	90	60	90	90
TG 24/84	90	50	50	50	70	90

a : log₁₀ 6 = 0.78

Long-dated and time consuming ecological developments will have probably to be considered to assess the possible significance of this factor in microbial antagonisms.

Acknowledgments

This work was supported by the grant of the Conseil Supérieur de la Pêche n° 2562 B.

Abstract

A soluble antimicrobial product has been detected in *Yersinia ruckeri* cultures. The contact droplets technique and the double agar layer method allowed to assess its bacteriostatic effect and the high susceptibility of other bacterial fish pathogens like *Vibrio* and *Aeromonas*. But it did not inhibit other strains of *Y. ruckeri*. No correlation was found between the antimicrobial activity and the virulence of the strains. As mixed infections with *A. salmonicida* and *Y. ruckeri* could be obtained experimentally, the factor does not seem to confer any competitive advantage in fish tissues colonization.

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