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Original research paper

Motility, biofilm, and endotoxin in *Ralstonia pickettii* isolates obtained from purified and ultrapure pharmaceutical water systems

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ABSTRACT

This study aimed to examine the motility, biofilm production, endotoxin release, and antibiotic resistance of 81 *Ralstonia pickettii* isolates collected from different pharmaceutical water systems in Croatia. Swimming and twitching motility were detected in all isolates, while swarming was not observed. Biofilm production was detected in approximately 40 % of the isolates under the tested conditions. Notably, extracellular polymeric substance (EPS) production was a common trait among all isolates. Endotoxin production was detected with the Limulus Amoebocyte Lysate test. Antibiotic susceptibility testing revealed consistent resistance to colistin, as well as significant resistance rates to β-lactam antibiotics, ertapenem, amoxicillin/clavulanic acid, ticarcillin and ampicillin. High susceptibility to first-generation cephalosporins, cephalexin, cefoxitin and chloramphenicol was observed. All isolates were susceptible to tigecycline and tetracycline. The isolates were grouped into three genetically closely related clusters, yet notable phenotypic diversity in biofilm production and antibiotic susceptibility persisted within these groups. The study highlights *R. pickettii*'s adaptability in pharmaceutical water systems, marked by its motility, biofilm-forming capabilities, and multidrug resistance. These results emphasise the importance of rigorous monitoring of water systems to reduce transmission risks and prevent the emergence of resistant strains in clinical environments.

Keywords: Ralstonia pickettii, motility, biofilm, endotoxin, resistance

INTRODUCTION

Ralstonia pickettii belongs to the genus *Ralstonia* and considering the results of the average nucleotide identity, it is considered that there are at least two subspecies, possibly two species of *R. pickettii* (1). It is often isolated from pharmaceutical industrial water production systems, where it forms biofilm. *R. pickettii* is an opportunistic pathogen, it colonises the hospital environment and patients, and it is associated with a wide range of hospital infections, often associated with the use of contaminated solutions that were declared sterile (2–11). Treatment of infection caused by *R. pickettii* can be a problem due to

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incomplete knowledge of the antibiotic resistance profile of this environmental bacterium (12).

Due to the frequent isolation of *R. pickettii* in samples obtained from ultrapure and purified pharmaceutical water systems during routine control, and a lack of relevant data for this geographic region, we collected and studied isolates of *R. pickettii* from different pharmaceutical industrial plants in two different areas of Croatia.

In our previous study, it was found that the most common aminoglycoside antibiotic resistance profile (85.2 %) was tobramycin-gentamicin-amikacin-netilmicin (T-G-A-N). The genes $bla_{\text{OXA-22}}$ and $bla_{\text{OXA-60}}$ were detected in 37.0 % and 80.3 % of isolates, respectively. Using pulsed-field gel electrophoresis, it was observed that the tested isolates were highly related (13).

In this study, we analysed the motility of isolates and the production of extracellular polymeric substance (EPS) as prerequisites for biofilm production, we examined the ability of isolates to form biofilm, and we expanded the antibiotic susceptibility testing to ten antibiotics that were not tested previously to obtain a complete resistance profile. Finally, we tested the endotoxin in selected isolates with different antibiotic resistance profiles and different biofilm production.

EXPERIMENTAL

Bacterial isolates

Eighty-one (81) isolates of *R. pickettii* were collected from five different plants for laboratory purified water (LPW) and pharmaceutical ultrapure water (UPW) from two different areas of Croatia (Table I).

According to the European pharmacopoeia, R2A agar (bioMérieux, France), intended for the cultivation of microorganisms from areas with low nutrients, was used for the cultivation of UPW samples, and tryptic soy agar (TSA, bioMérieux) was used for the cultivation of microorganisms from LPW. Columbia blood agar (COL, bioMérieux) was used for subcultivation. A commercial biochemical test for non-fermentative bacteria was used for identification, and all isolates identified as *R. pickettii* were confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and by polymerase chain reaction, as described in our previous study (13).

Testing the antibiotic susceptibility of R. pickettii *isolates*

The antibiotic susceptibility of all *R. pickettii* isolates was tested by Kirby-Bauer disc diffusion method (DD) on Mueller Hinton agar (MHA, bioMérieux) against 10 antibiotics with antimicrobial discs (MASTDISCS®AST, MastGroup, UK) of ampicillin (AMP 20 μg), amoxicillin/clavulanic acid (AMC 20/10 μg), cephalexin (CN 30 μg), cefoxitin (FOX 30 μg), ertapenem (ETP 10 μg), ticarcillin (TIC 75 μg), tetracycline (TE 30 μg), tigecycline (TGC 15 μg), colistin (CL 50 μg) and chloramphenicol (C 30 μg). The results were read and interpreted according to CLSI guidelines for *Pseudomonas* spp, *Acinetobacter* spp, *Burkholderia* spp, *Enterobateriaceae* (15) and European Committee on Antibacterial Susceptibility Testing guidelines for colistin for *Acinetobacter* spp.

Table I. Ralstonia pickettii *isolates from two areas in Croatia (I and II) and five pharmaceutical water plants/sources (Pm, Pp, HL, G and K)*

Area of isolates	Industrial plant designation		Isolate	Source	No of isolates
I	P	Pm	*R. pickettii* Pm8, Pm20, Pm68, Pm79, Pm80, Pm81, Pm88, Pm89, Pm90, Pm91, Pm92, Pm95, Pm96, Pm104, Pm373, Pm374, Pm375, Pm376, Pm379, Pm381, Pm382, Pm383, Pm384, Pm385, Pm386, Pm387, Pm388, Pm389, Pm390	LPW	29
		Pp	*R. pickettii* Pp78, Pp83, Pp94, Pp377, Pp378, Pp391	LPW	6
	HL		*R. pickettii* HL15, HL16, HL17, HL18, HL19, HL21, HL22, HL23, HL24, HL25, HL26, HL27, HL28, HL29, HL30, HL31, HL32, HL33, HL34, HL35, HL36, HL93, HL392, HL393, HL394, HL395, HL398, HL399, HL100,	UPW	29
	GL		*R. pickettii* GL13, GL14, GL54, GL55, GL56, GL77, GL103	LPW	7
II	K		*R. pickettii* K82-1, K82-2, K84, K85, K97, K98, K99, K100, K101, K102	UPW	10
Total					81

LPW – laboratory purified water, UPW – ultrapure water

Motility testing

Different motility types of *R. pickettii* were tested by seeding on nutrient media with different agar concentrations at 37 °C (16, 17). Swimming motility was tested in sulphide indole motility nutrient medium (SIM, Becton, Dickinson and Company, USA) in a test tube, and in a Petri plate with Luria Bertani nutrient medium (LB, Becton, Dickinson and Company) with 0.3 % agar. Motility in a test tube was manifested by turbidity, and the distance of turbidity from the puncture line in mm was measured after cultivation for 24 and 72 hours. The diameter of bacterial migration from the seeding site on LB agar was measured after 2, 24, 72 and 96 hours of incubation. Swarming motility was tested on 0.5–0.7 % LB agar, and twitching on 1 % LB agar. For the twitching assay, the isolate was inoculated by pricking the agar surface at the bottom of a Petri dish. The diffuse zone of bacterial growth between the agar surface and the bottom of the Petri dish, measured after 24 hours, was a measure of twitching motility. All tests were performed at least in two independent experiments.

Production of extracellular polymeric substances

EPS production in *R. pickettii* was tested by cultivation in liquid nutrient broth on perlite balls and on aluminium foil, in liquid tryptic soy broth (TSB, bioMérieux) without additions of other supports and on a solid surface of R2A agar (Oxoid, UK). After cultivation for 48 hours at 37 °C, direct smears were prepared from bacterial deposits on perlite and from aluminium foil and stained, first with alcian blue, and then fuchsin. Alcian blue

stained the EPS, and fuchsin visualised the bacterial cell by staining it red (17). Microscopic slides from TSB and from R2A were prepared from the grown cultures and stained according to Gram. All tests were performed at least in two independent experiments.

Biofilm production

Biofilm production was examined by the microtiter plate assay, and the quantification of produced biofilm was performed by crystal violet staining, and optical density (OD) of the discoloured biofilm was measured spectrophotometrically at a wavelength of 620 nm (18). All isolates were tested for biofilm production in two independent experiments with four technical replicates, after incubation for 4 h, 6 h, 8 h and 24 h at 37 °C. Seven strains which were less mobile in the test tube motility and with different swimming type motility were incubated for an additional 24 hours (48 hours in total). The isolates with OD values higher than the OD of the negative control were considered biofilm producers. The optical density cut-off value (ODc) was used to categorise biofilm producers and defined by the formula: $ODc = averageOD_{negative\ control} + (3\times SD_{negative\ control})$. The OD value for each isolate was calculated according to the formula: $OD_{isolate} = meanOD_{isolate} - ODc$. According to the obtained OD results, the isolates were categorised as: $OD < ODc$, non-biofilm producer (NBP); $ODc < OD < 2\times ODc$, weak biofilm producer (WBP); $2\times ODc < OD < 4\times ODc$, moderate biofilm producer (MBP), and $OD > 4\times ODc$, strong biofilm producer (SBP) (19).

Endotoxin detection

Endotoxin was examined with the Limulus Amoebocyte Lysate test (LAL) (20, 21). We tested 6 isolates from different areas of Croatia with different phenotypes of resistance to aminoglycosides and different biofilm production. Samples for endotoxin assay were prepared by autoclaving a bacterial suspension of 10^6 CFU mL^{-1} at 136 °C to destroy the bacterial cells and release the present endotoxin. Control standard endotoxin (CSE) was used in concentrations 2λ, 1λ, 0.5λ, and 0.25λ. Lambda is the endotoxin concentration required to form a gel in the reaction with the lysate and indicates the sensitivity of the lysate. In our test, λ was 0.0625 EU mL^{-1}. After the end of incubation, each test tube was carefully turned 180°, and the presence of endotoxins was determined based on the formation of a solid gel. At the same time, the release of endotoxin in the standard strain *E. coli* ATCC 25922 was used as a positive control. The test was performed in two independent experiments.

Genetic relatedness of R. pickettii *isolates*

The relatedness of all identified *R. pickettii* isolates from different sources/locations in Croatia was investigated by pulsed field gel electrophoresis (PFGE), and a computerised dendrogram of DNA profile similarity was created in our previous study (13).

Statistical analysis

Statistical analysis of biofilm production related to different aminoglycoside resistance profiles was determined by the Fisher's exact test as described in Mayhua 2019. A *t*-test was used to analyse the significance of the swimming motility during different times and biofilm production (p-value < 0.05 considered statistically significant).

RESULTS AND DISCUSSION

We tested *R. pickettii* isolates that were collected during regular quality monitoring of ultrapure and purified water systems located in different areas of Croatia. This study was initiated due to the assumption that the almost constant occurrence of *R. pickettii* in our water samples from controlled industrial and laboratory facilities would result in the existence of biofilms in these systems. After cultivation for 18–24 hours at 37 °C, colonies of *R. pickettii* grew on membrane filters on TSA, R2A as shiny, moist, slightly convex, light brown and/or brown to dirty pink, with a diameter of 0.5–2 mm, and on blood agar they grew as shiny grey colonies. Small, delicate Gram-negative rods, 0.8–2 μm × 0.3 μm in size, were seen in the Gram-stained microscopic preparation (Fig. 1).

Motility property and biofilm production

In the previous studies, the biofilm and the properties required for biofilm production, such as twitching motility and EPS production, were studied on a very limited number of isolates (22, 23). Our analysis was performed on a large collection of 81 isolates, and we extended it to three different motility types – swimming (in a test tube and on the agar surface), twitching, and swarming. All isolates showed swimming motility in the test tube, and different forms of turbidity were observed, from uniform to irregular to fan-shaped (Fig. 2a). On the first day of cultivation, 67 % isolates grew 3 mm or more from the puncture line (Table II). Most isolates had a significantly increased initial turbidity after the third day of incubation because the cells, due to their motility, inhabited areas further from the puncture line. All isolates also showed swimming motility on Luria Bertani nutrient

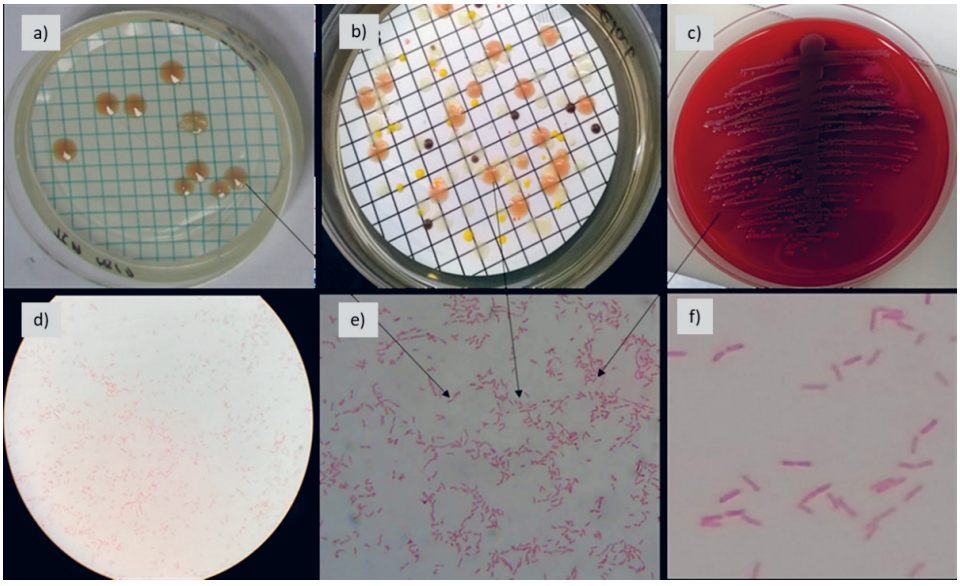

Fig. 1. *R. pickettii* light brown/dirty pink colonies on membrane filter: a) pure culture; b) mixed culture on Tryptic soy agar; c) pure culture of grey, moist colonies on Columbia blood agar. Gram-negative rods in a microscopic preparation with magnification: d) 1000×; e) 2000×; f) 4000×.

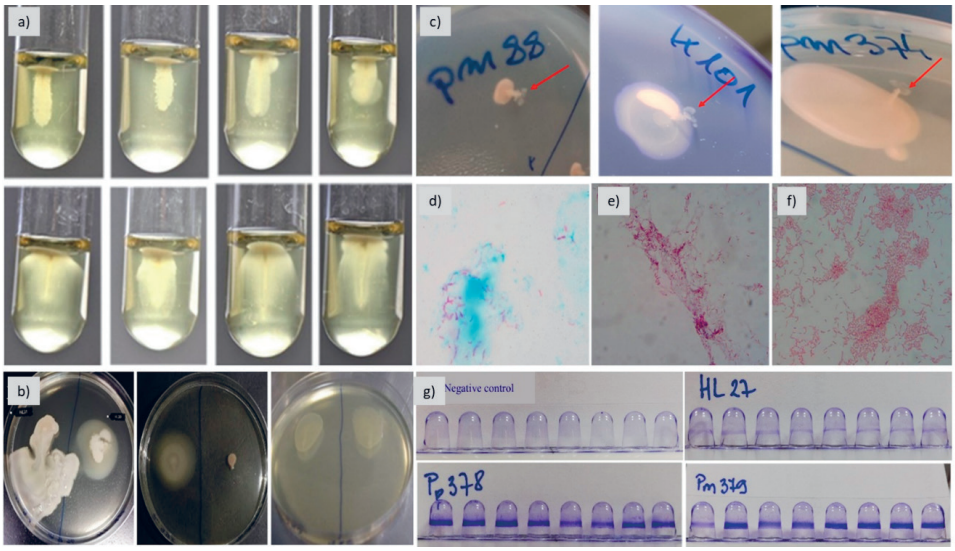

Fig. 2. Motility of *R. pickettii* in test tube: a) eight representative samples showing different forms of turbidity; b) swimming motility of representative samples of *R. pickettii* isolates on agar surfaces; c) twitching motility. Microscopic appearance of *R. pickettii* and EPS: d) cultured on perlite and stained with alcian blue, EPS-blue, bacterial cells-red; e) cultured in TSB and stained by Gram, EPS-red, cells-red; f) cultured on the surface of R_2A agar, without EPS, only cells red; g) negative control and test results of biofilm production in HL27, Pp378 and Pm379 isolates.

medium (Fig. 2b), 87 % already after two hours of cultivation. The majority of isolates reached a greater distance from the seeding place with longer incubation. All isolates showed twitching motility necessary for biofilm production (Fig. 2c), while none of them showed the swarming motility.

Production of extracellular polymeric substances (EPS)

R. pickettii produced the EPS on perlite in TSB broth and in TSB, but not on aluminium foil or on a solid nutrient medium. Microscopic preparations made from the deposits formed on perlite beads clearly showed red-stained bacterial cells and blue-stained areas representing the EPS formed by the cells after 48 hours of cultivation (Fig. 2d). There were no visible traces of EPS in the aluminium foil preparations. In the Gram preparation from TSB, red-stained bacterial cells and red-stained mucous material around the cells were clearly visible (Fig. 2e), while in the preparation from a solid substrate, only red-stained bacterial cells were visible (Fig. 2f).

Biofilm production

Although all our isolates produced EPS and showed the motility types required for biofilm production, we did not demonstrate biofilm production in all isolates under our testing conditions. Of the 81 tested isolates, 6.2 % were MBP, 30.9 % were WBP, and 63 % of isolates did not produce biofilm (NBP) (Table II, Fig. 3).

Table II. Motility properties and biofilm production of R. pickettii *isolates compared to their aminoglycoside resistance phenotype*

Isolate	Motility in the test tube (mm) after		Swimming motility on 0.3 % TSA agar (mm) after				Twitching motility	Biofilm production after		Aminoglycoside resistance phenotype
	24 h	72 h	2 h	24 h	72 h	96 h		24 h	48 h	T-G-A-N
Pm8	3	5	1	20	25	30	+	NBP		R-R-R-R
GL13	1.5	1.5	1	22	30	30	+	NBP		R-R-R-R
GL14	3	11	1	2	5	10	+	WBP		R-R-R-R
HL15	6	12	3	6	15	29	+	NBP		R-R-R-R
HL16	4	11	3	9	15	30	+	NBP		R-R-R-R
HL17	1	1	1	7	17	28	+	NBP		R-R-R-R
HL18	4	10	0	30	30	30	+	NBP		R-R-R-R
HL19	3	4	0	30	30	30	+	NBP		R-R-R-R
Pm20	5	11	2	5	18	45	+	NBP		R-R-R-R
HL21	5	11	1.5	5	10	24	+	NBP		R-R-R-R
HL22	2	2	1.5	5	17	29	+	NBP		R-R-R-R
HL23	5	13	1	30	30	30	+	NBP		R-R-R-R
HL24	6	12	2.5	10	16	20	+	NBP		R-R-R-R
HL25	4	6	0	30	30	30	+	NBP		R-R-R-R
HL26	4	6	1	5	12	25	+	NBP		R-R-R-R
HL27	2	2	1	10	17	29	+	NBP	NBP	R-R-R-R
HL28	1.5	1.5	1.5	3	6	12	+	WBP		S-S-R-R
HL29	5	7	1.5	4	9	15	+	NBP		R-R-R-R
HL30	1.5	1.5	1	9	15	32	+	NBP	WBP	S-S-R-R
HL31	6	10	2	30	30	30	+	NBP		R-R-R-R
HL32	3	4	1	7	12	18	+	NBP	NBP	R-R-R-R
HL33	5	8	1	4	9	15	+	WBP		R-R-R-R
HL34	5	12	1	5	13	25	+	NBP		R-R-R-R
HL35	3	5	0	3	7	14	+	NBP		R-R-R-R
HL36	2	3	1	4	7	11	+	NBP		R-R-R-R
GL54	3	11	1	5	8	18	+	MBP		R-R-R-R
GL55	1.5	2	2	4	9	15	+	NBP		R-R-R-R
GL56	3	10	2	6	10	23	+	NBP		R-R-R-R
Pm68	2	3	3	11	12	14	+	NBP		R-R-R-R
GL77	3	7	0	12	16	20	+	NBP		R-R-R-R
Pp78	3	4	1.5	6	9	11	+	NBP		R-R-R-R
Pm79	3	4	2	9	10	12	+	WBP		R-R-R-R
Pm80	3	3	2.5	5	30	30	+	NBP		R-R-R-R
Pm81	3	4	1.5	5	9	12	+	NBP		R-R-R-R

Isolate	Motility in the test tube (mm) after		Swimming motility on 0.3 % TSA agar (mm) after				Twitching motility	Biofilm production after		Aminoglycoside resistance phenotype
	24 h	72 h	2 h	24 h	72 h	96 h		24 h	48 h	T-G-A-N
K82-1	2	4	2	10	12	18	+	NBP		S-S-S-R
K82-2	2	3	2	12	15	24	+	NBP		S-R-R-R
Pp83	3	6	4	4	6	9	+	WBP		R-R-R-R
K84	1.5	1.5	4	6	10	25	+	WBP		S-S-R-R
K85	3	11	2.5	7	15	29	+	NBP		S-R-R-R
Pm88	3	4	0	3	6	9	+	NBP		R-R-R-R
Pm89	3	7	3	4	7	16	+	NBP		R-R-R-R
Pm90	2	3	5	5	7	10	+	NBP		R-R-R-R
Pm91	3	3	3	3	8	20	+	WBP		R-R-R-R
Pm92	6	12	12	12	15	25	+	WBP		R-R-R-R
HL93	5	12	1.5	5	12	21	+	NBP		R-R-R-R
Pp94	6	8	2	6	10	15	+	NBP		R-R-R-R
Pm95	5	10	2	3	7	9	+	NBP		R-R-R-R
Pm96	1.5	1.5	3	10	30	30	+	NBP		R-R-R-R
K97	1.5	1.5	0	5	9	20	+	WBP		S-S-S-S
K98	3	10	2.5	10	15	29	+	WBP		S-S-R-S
K99	2	2	2.5	7	11	25	+	WBP		S-S-S-S
K100	2	5	0	5	9	16	+	NBP	WBP	S-S-S-S
K101	2	3	1.5	5	10	20	+	WBP		S-S-S-S
K102	1.5	1.5	0	10	30	30	+	WBP		R-S-S-S
GL103	1.5	2	0	7	10	15	+	WBP	MBP	R-R-R-R
Pm104	6	12	1	6	11	22	+	NBP		R-R-R-R
Pm373	2	2	2	7	9	15	+	WBP		R-R-R-R
Pm374	2	3	2	30	30	30	+	WBP		R-R-R-R
Pm375	6	12	2	6	15	40	+	NBP		R-R-R-R
Pm376	6	11	0.5	8	30	30	+	NBP		R-R-R-R
Pp377	3	3	2.5	7	10	15	+	WBP		R-R-R-R
Pp378	3	4	2	6	7	11	+	WBP	MBP	R-R-R-R
Pm379	3	5	0.5	7	8	13	+	WBP	MBP	R-R-R-R
Pm381	3	4	3	6	9	15	+	NBP		R-R-R-R
Pm382	3	3	2	5	7	12	+	WBP		R-R-R-R
Pm383	3	3	2	7	8	10	+	WBP		R-R-R-R
Pm384	4	7	2.5	7	7	11	+	MBP		R-R-R-R
Pm385	8	12	2	17	32	45	+	MBP		R-R-R-R
Pm386	3	3	1	30	30	30	+	MBP		R-R-R-R
Pm387	1.5	3	1.5	5	7	10	+	WBP		R-R-R-R

Isolate	Motility in the test tube (mm) after		Swimming motility on 0.3 % TSA agar (mm) after				Twitching motility	Biofilm production after		Aminoglycoside resistance phenotype
	24 h	72 h	2 h	24 h	72 h	96 h		24 h	48 h	T-G-A-N
Pm388	3	11	2	25	30	30	+	MBP		R-R-R-R
Pm389	3	4	2.5	6	8	10	+	WBP		R-R-R-R
Pm390	1.5	1.5	2	7	8	12	+	WBP		R-R-R-R
Pp391	2	2	3	10	10	15	+	WBP		R-R-R-R
HL392	5	10	2.5	9	14	20	+	NBP		R-R-R-R
HL393	1.5	1.5	3	9	15	25	+	NBP		R-R-R-R
HL394	5	11	2	5	9	10	+	NBP		R-R-R-R
HL395	1.5	1.5	2	30	30	30	+	NBP		R-R-R-R
HL398	3	4	2	6	10	10	+	NBP		R-R-R-R
HL399	4	11	0	30	30	30	+	NBP		R-R-R-R
HL100	3	3	2	15	16	20	+	NBP		R-R-R-R

NBP – non-biofilm producer (OD < ODc), WBP – weak biofilm producer (ODc < OD < 2× ODc), MBP – moderate biofilm producer (2× ODc < OD < 4× ODc), T-G-A-N – tobramycin-gentamicin-amikacin-netilmicin, S – susceptible, R – resistant

We observed that some isolates that did not form biofilm still left a visible stain on the well walls (Fig. 2g), so we extended the incubation time to 48 hours for the seven selected isolates (HL27, HL30, HL32, K100, GL103, Pp378, Pm379) to examine whether the status of the biofilm producers would change over time. No change occurred in two isolates (HL27 and HL32). These isolates kept their original status as non-biofilm producers, whereas two isolates (HL30 and K100) changed from non-producers to weak biofilm producers, and three isolates (GL102, Pp378 and Pm379) changed from weak producers to moderate biofilm producers. The values of optical density throughout the observed time for isolates that produce biofilm indicate that there is an early phase of growth up to 6 to 8 hours, which then falls and then grows again up to 48 hours (Fig. 4).

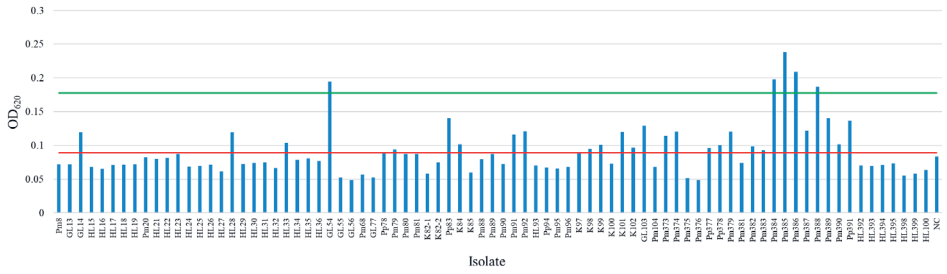

Fig. 3. Biofilm production of *R. pickettii* isolates after 24 h of incubation. Below the red line are non-biofilm producers (OD < 0.089), between the red and green lines are weak biofilm producers (OD 0.089–0.176), and above the green line are moderate biofilm producers (OD ≥ 0.177). NC – negative control (medium without bacteria).

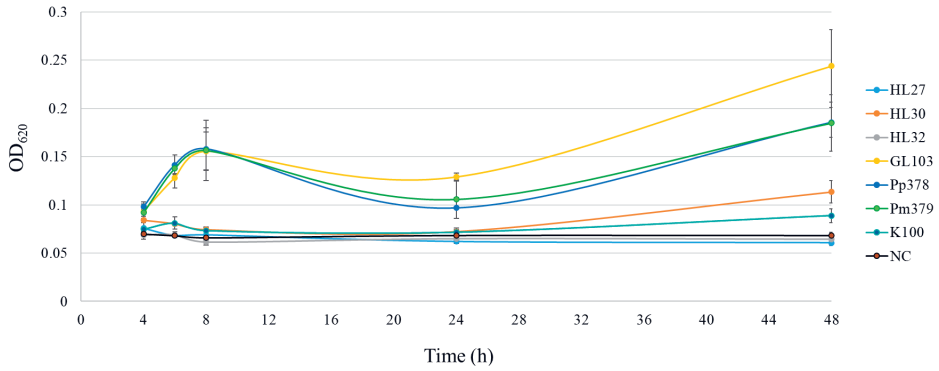

Fig. 4. Biofilm development of representative isolates in 48 hours. NC – negative control; error bars – standard deviation.

If we had changed the conditions of the assay itself, for example, by growing in a system with refreshing bacterial nutrient medium or perhaps by extending the cultivation time, we might have achieved better biofilm production in the isolates that left a visible stain on the well wall. There was no indication that variations in the swimming motility and the motility in the test tube influenced the production of biofilm No statistically significant difference in test tube motility was obtained between non biofilm producers and biofilm producers, regardless of the length of incubation ($p_{\text{first day}} = 0.062$, $p_{\text{third day}} = 0.052$) or in swimming on the agar surface after 2 hours ($p = 0.178$). Significantly larger swimming radii were observed in non-biofilm producers in the first 24 hours than after 96 hours of incubation ($p = 0.013$).

Endotoxin detection

Investigated isolates with different aminoglycoside resistance and biofilm production profiles produced endotoxin in concentrations close to the concentration of endotoxin in the control *E. coli* strain (Table III).

The structure of its LPS decreases cytokine levels (24), is consistent with the low-grade inflammation in obese patients (14), and is associated with worsened glucose intolerance in obesity (25), suggesting that *R. pickettii* could turn from a common environmental bacterium and opportunistic pathogen into a problematic hospital strain. This is supported by severe *R. pickettii* infections that were described in several individual cases, some of which were very serious (6, 7, 26). Thus far, *R. pickettii*-related infections have not been documented in Croatia.

Antibiotic resistance profiles

High rates of resistance for aztreonam, four aminoglycosides (netilmicin, amikacin, tobramycin, gentamicin) and quite high resistance rates for ticarcillin/clavulanic acid and β-lactam antibiotics for the same isolates were detected in our previous study (13). Here we detected 100 % resistance rate to colistin, as well as significant resistance rates to β-lactam antibiotics: 91.3 % of the isolates were resistant to ertapenem, 71.6 % were resistant to

Table III. Endotoxin concentrations in control E. coli *strain and* R. pickettii *isolates obtained by the LAL method*

Sample dilution	Isolates							Endotoxin concentration (EU mL^{-1})
	E. coli	Pm 20	HL 27	HL 30	K 85	K 99	GL 103	
original	+	+	+	+	+	+	+	0.0625
1:2	+	+	+	+	+	+	+	0.125
1:4	+	+	+	+	+	+	+	0.25
1:8	+	+	–	+	+	+	–	0.5
1:16	–	+	–	+	–	+	–	1
1:32	–	+	–	+	–	–	–	2
1:64	–	–	–	–	–	–	–	4

positive reaction (+), negative rection (–)

amoxicillin/clavulanic acid, 67.9 % to ticarcillin and 58.0 % to ampicillin. In addition to the already described high sensitivity of *R. pickettii* isolates to the cephalosporin antibiotics ceftriaxone, cefotaxime, cefepime and ceftazidime (13), in this study, we found a very high sensitivity to the first-generation cephalosporins cephalexin (96.3 %) and cefoxitin (97.5 %). All isolates were susceptible to tigecycline and tetracycline (Table IV).

The antibiotic resistance profiles of tested isolates from this study, as well as the results obtained in our previous study (13), revealed discrete differences in comparison to the observations published by other authors. We summarised our results and data found in the literature in Table V.

Table IV. Resistance profile of R. pickettii *isolates obtained by the disc diffusion method*

Antibiotic[a]	Zone diameter			Disc (μg)	*Ralstonia pickettii* (n = 81)		
	R	I	S		R	I	S
					No (%)	No (%)	No (%)
Ampicillin	≤ 13	14–16	≥ 17	10	47 (58.0 %)	20 (24.7 %)	14 (17.3 %)
Amoxicillin / clavulanic acid	≤ 13	14–17	≥ 18	20/10	58 (71.6 %)	17 (20.0 %)	6 (7.4 %)
Ticarcillin	≤ 15	16–23	≥ 24	75	55 (67.9 %)	24 (29.6 %)	2 (2.5 %)
Ertapenem	≤ 15	16–18	≥ 19	10	74 (91.3 %)	5 (6.2 %)	2 (2.5 %)
Cefalexin	≤ 19	20–22	≥ 23	30	2 (2.5 %)	1 (1.2 %)	78 (96.3 %)
Cefoxitin	≤ 14	15–17	≥ 18	30	2 (2.5 %)	0	79 (97.5 %)
Tigecycline	≤ 11	12–14	≥ 15	30	0	0	81 (100 %)
Tetracycline	≤ 11	12–14	≥ 15	30	0	0	81 (100 %)
Chloramphenicol	≤ 12	13–17	≥ 18	30	7 (8.6 %)	33 (40.7 %)	41 (50.6 %)
Colistin	≤ 10	–	≥ 11	50	81 (100 %)	0	0

[a] Inhibition zones for *P. aeruginosa*, *Acinetobacter* spp and enterobacteria were used (CLSI, 2018, EUCAST 2019); R – resistant, I – intermediate, S – susceptible

To make a better comparison of our results on isolates from purified and ultrapure water systems, we separated them from results published by other authors on clinical and environmental isolates from different water sources. It is disturbing that *R. pickettii* is almost fully resistant to colistin, both in clinical settings and in different water sources, but fortu-

Table V. Antimicrobial resistances identified in 81 R. pickettii *isolates from water systems in Croatia (this work and (13) and in different* R. pickettii *isolates (clinical settings and different water sources) obtained from a literature review*

Antibiotic	*R. pickettii* – water sources in Croatia	*R. pickettii* – clinical strains (previous studies)[a]	*R. pickettii* – water sources (previous studies)[b]
Ampicillin	47/81 (58.0 %)	2/4 (50.0 %)	22/23 (95.7 %)
Amoxicillin/clavulanic acid	58/81 (71.6 %)	1/1 (100 %)	0/1 (0 %)
Ticarcillin	55/81 (67.9 %)	not tested	18/37 (48.6 %)
Ticarcillin/clavulanic acid	50/81 /61.7 %)	not tested	not tested
Piperacillin	2/81 (2.5 %)	1/1 (100 %)	0/20 (0 %)
Piperacillin/tazobactam	2/81 (2.5 %)	2/17 (11.8 %)	0/15 (0 %)
Ertapenem	74/81 (91.4 %)	1/1 (100 %)	not tested
Imipenem	0/81 (0 %)	**8/21 (38.1 %)**	0/17 (0 %)
Meropenem	**77/81 (95.0 %)**[c]	13/19 (68.4 %)	9/51 (17.6 %)
Aztreonam	**78/81 (96.3 %)**	16/18 (88.9 %)	not tested
Cefalexin	2/81 (2.5 %)	2/4 (50.0 %)	1/1 (100 %)
Cefoxitin	2/81 (2.5 %)	0/1 (0 %)	1/21 (4.8 %)
Ceftriaxone	0/81 (0 %)	0/2 (0 %)	0/17 (0 %)
Ceftazidime	11/81 (13.6 %)	**11/25 (44.0 %)**	19/71 (26.8 %)
Cefotaxime	0/81 (0 %)	1/2 (50.0 %)	0/20 (0 %)
Cefepime	0/81 (0 %)	2/11 (18.2 %)	not tested
Gentamicin	70/81 (86.4 %)	13/15 (86.7 %)	56/61 (91.8 %)
Amikacin	**71/81 (87.7 %)**	11/18 (61.1 %)	4/15 (26.7 %)
Tobramycin	72/81 (88.9 %)	11/14 (78.6 %)	8/9 (88.9 %)
Netilmicin	72/81 (88.9 %)	0/1 (0 %)	not tested
Tigecycline	0/81 (0 %)	0/2 (0 %)	0/14 (0 %)
Tetracycline	0/81 (0 %)	0/5 (0 %)	0/40 (0 %)
Trimethoprim/sulfamethoxazole	0/81 (0 %)	1/22 (4.5 %)	0/52 (0 %)
Ciprofloxacin	2/81 (2.5 %)	0/15 (0 %)	1/71 (1.4 %)
Chloramphenicol	7/81 (8.6 %)	1/1 (100 %)	2/3 (66.7 %)
Colistin	81/81 (100 %)	14/15 (93.3 %)	65/65 (100 %)

[a] References for *R. pickettii* clinical strains (1, 3, 4, 6, 8, 12, 27, 30, 31). [b] References for *R. pickettii* from various water sources (11, 28, 32, 33). [c] Distinct differences are indicated in bold.

nately it remains highly susceptible to piperacillin, piperacillin/tazobactam, imipenem (in water systems), cefoxitin, ceftriaxone, cefotaxime, tigecycline, tetracycline, trimethoprim/sulfamethoxazole and ciprofloxacin. Clinical strains exhibit increased resistance to imipenem and ceftazidime, indicating the spreading of resistance genes by horizontal transfer in hospital environments. It is interesting, however, that isolates from Croatian water systems are highly resistant to all tested aminoglycosides (T-G-A-N), whereas clinical strains and especially other previously analysed water sources isolates show a lower resistance rate to amikacin. Similar results are noted also for meropenem and aztreonam, indicating local spread of carbapenemase genes, as confirmed by our previous study (13).

Genetic relatedness of R. pickettii *isolates*

In our previous study, we determined the relatedness of *R. pickettii* isolates by PFGE (13), and here we analysed the obtained clusters with regard to the biofilm production and aminoglycoside resistance phenotype. We showed previously that three main clusters with a different number of subclusters were formed by hierarchical grouping; most isolates (57.0 %) were in cluster A, 8.9 % of isolates in cluster B and 35.4 % isolates in cluster C (Table VI).

Table VI. Three clusters and their subclusters formed by R. pickettii *isolates from specific areas, according to biofilm production and aminoglycoside resistance phenotype*

Cluster	Subcluster	Isolates												
		Total (n = 79)	GL (n = 7)	Pm (n = 27)	Pp (n = 6)	HL (n = 29)	K (n = 10)	NBP (n = 48)	WBP (n = 24)	MBP (n = 7)	TGAN R (n = 67)	TGAN S (n = 9)	N R (n = 2)	T R (n = 1)
A	1.	11	0	10	1	0	0	1	7	3	11	0	0	0
	2.	13	2	5	3	2	1	6	5	2	10	1	2	0
	3.	7	0	0	0	0	7	3	4	0	0	7	0	0
	4.	5	2	2	0	1	0	4	1	0	5	0	0	0
	5.	9	3	4	0	0	2	3	4	2	7	1	0	1
	Total	45	7	21	4	3	10	17	21	7	33	9	2	1
B	1.	3	0	2	1	0	0	2	1	0	3	0	0	0
	2.	1	0	0	1	0	0	1	0	0	1	0	0	0
	3.	1	0	1	0	0	0	1	0	0	1	0	0	0
	4.	1	0	1	0	0	0	0	1	0	1	0	0	0
	Total	6	0	4	2	0	0	4	2	0	6	0	0	0
C	1.	16	0	1	0	15	0	16	0	0	16	0	0	0
	2.	4	0	0	0	4	0	3	1	0	4	0	0	0
	3.	8	0	1	0	7	0	8	0	0	8	0	0	0
	Total	28	0	2	0	26	0	27	1	0	28	0	0	0

GL, Pm, Pp, HL – isolates from sampling sites in the area I; K – isolates from sampling sites in the area II. NBP – non biofilm producer, WBP – weak biofilm producer, MBP – moderate biofilm producer, TGAN – tobramycin, gentamicin, amikacin, netilmicin; N – netilmicin, T– tobramycin, R – resistant; S – susceptible

We attempted to correlate the ability to produce biofilm with the geographic distribution of isolates, but our biofilm producers were equally distributed in both Croatian areas and in all five sources. All three clusters contained both biofilm producers and non-biofilm producers. In cluster A, approximately 50 % of isolates showed aminoglycoside resistance phenotype T-G-A-N (33/67). There were two isolates susceptible to tobramycin, amikacin, and gentamicin and resistant to netilmicin, and 9/79 isolates were susceptible to all four aminoglycoside antibiotics, all were from area II (K isolates). All isolates in clusters B and C had the same phenotype, T-G-A-N.

CONCLUSIONS

R. pickettii is an opportunistic bacterium that contaminates pharmaceutical production plants and the hospital environment and may cause infections, sometimes with a fatal outcome. Our study highlights *R. pickettii*'s adaptability in Croatian pharmaceutical water systems, marked by its motility, biofilm-forming capabilities, production of endotoxin and multidrug resistance. *R. pickettii* can be a source of resistance genes for other microorganisms it meets – it can also develop new resistance mechanisms due to innate potential, due to selection pressure or by exchanging genetic material with other bacteria. *R. pickettii* should therefore be regarded as a potential causative agent of nosocomial infections, necessitating careful consideration to ensure the administration of appropriate therapeutic interventions, prevent the emergence of antibiotic-resistant strains, and minimise its survival in clean and ultra-clean water systems.

Ethical approval statement. – Ethical approval was deemed unnecessary for this study, as the bacterial isolates were obtained exclusively from various water sources and did not involve the collection of samples from human or animal subjects.

Data availability statement. – All data generated or analysed during this study are included in this published article.

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