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Detection methods for carbapenem-resistant *Pseudomonas aeruginosa* in surface water and wastewater

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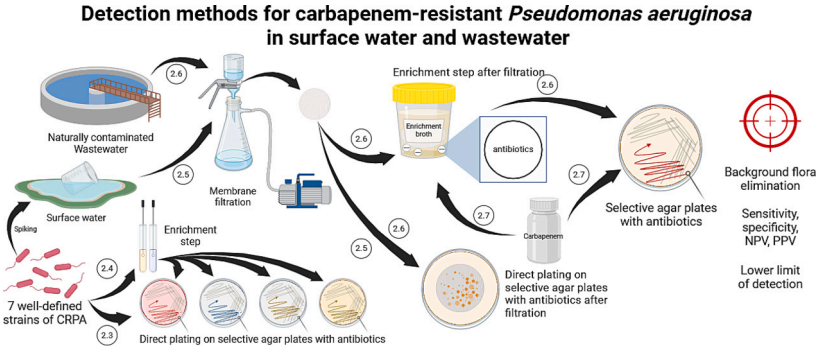
HIGHLIGHTS

- A standardized method for detecting CRPA in surface water and wastewater samples by culture has been developed.
- Enrichment is recommended for maximum specificity.
- Imipenem is recommended as selective agent in a combination of nonselective enrichment and selective plating.
- Filtration is recommended as it enables concentrating the bacteria from a bigger volume of samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Water systems can act as an important reservoir for *Pseudomonas aeruginosa*, which can pose public health risks during human contact. Carbapenem resistance is one of the most concerning resistances in *P. aeruginosa* making it a high-priority pathogen according to the World Health Organization (WHO), due to its ability to cause difficult-to-treat infections. Culture methods for detecting carbapenem-resistant *P. aeruginosa* (CRPA) have generally been developed for clinical samples but rarely for water. Therefore, the aim of this study was to develop a standardized and widely applicable method for detecting CRPA in water samples, both in surface water and wastewater. Growth of well-characterized strains of CRPA was determined in four different selective agar media which were each supplemented with imipenem, meropenem, or ceftazidime, including after an enrichment. Wastewater samples presumptively containing CRPA (naturally contaminated samples) and surface water samples spiked with well-characterized CRPA strains were filtered and cultured using selective agar plates supplemented with imipenem and meropenem, with and without a prior enrichment step. M-PA-C agar plates supplemented with imipenem performed best compared to other selective agar plates with respect to growth of well-characterized CRPA strains and suppression of background growth of other bacteria and carbapenem-

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susceptible *P. aeruginosa* (CSPA) with positive predictive value of 80–100 %. A prior step using asparagine proline broth with vancomycin is recommended for higher specificity considering direct plating was unsuccessful due to extensive background growth. A combination of nonselective enrichment with selective plating (i.e., addition of imipenem to the plate) is recommended to achieve low limits of detection. The addition of imipenem in the broth instead of in solid agar plates eliminated some of the CRPA strains, producing an increase in the lower limit of detection and a lower PPV (32 % vs 73 %).

1. Introduction

Pseudomonas aeruginosa are non-fermentative Gram-negative bacilli which are ubiquitous in moist environments. Therefore, water systems can act as an important reservoir for *P. aeruginosa*, and this can lead to public health risks during human contact (Kanamori et al., 2016). In addition, its ability to form biofilm makes it more difficult to eradicate *P. aeruginosa* from water sources, since the protective layer prevents biocides from penetrating them (Bédard et al., 2016). A recent meta-analysis showed that *P. aeruginosa* can be found in 92 % of wastewater samples and 86 % of urban river samples (Crone et al., 2020). The concentrations of *P. aeruginosa* in natural waters have been determined to lie between 10 and 1000 CFU/mL (Mena and Gerba, 2009). Moreover, *P. aeruginosa* found in surface waters have been reported to be resistant to antibiotics, including carbapenems (Gad et al., 2007; Schiavano et al., 2017). This is concerning as carbapenem resistance is one of the most serious forms of resistance in *P. aeruginosa*, making it a high priority pathogen by the World Health Organization (WHO) due to its ability to cause severe infections, including pneumonia, bloodstream infections, and wound infections, which are often difficult-to-treat (*WHO Bacterial Priority Pathogens List, 2024: Bacterial Pathogens of Public Health Importance to Guide Research, Development and Strategies to Prevent and Control Antimicrobial Resistance*, 2024; Papadimitriou-Olivgeris et al., 2022; Bassetti et al., 2018). Consequently, carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) may pose a problem in hospital wastewater systems as it can contaminate the environment, potentially leading to the spread of antibiotic resistance and public health risks (Roulová et al., 2022).

The standard ISO method for detecting *P. aeruginosa* in water uses membrane filtration and selective culture media, such as Cetrimide agar, acetamide broth and King B agar, at 36 °C incubation (*ISO 13843:2017 Water Quality – Establishing Peformance Characteristics Quantitative Methods*, 2017; *ISO 16266:2006 Water Quality – Detection and Enumeration of Pseudomonas aeruginosa – Method by Membrane Filtration*, 2006). This method allows *P. aeruginosa* to be quantified, but has low sensitivity as well as several other limitations, such as being time-consuming and showing reduced selectivity and recovery rates in the presence of high competitive flora (Casanovas-Massana et al., 2010; Ngwa et al., 2017; Spies et al., 2020). Moreover, atypical colonies that do not produce pyocyanin might be missed (Sartory et al., 2015). Beside the ISO standard, some other media and enrichment broths, i.e. asparagine broth and King A agar, were also widely used for detecting *P. aeruginosa* (Casanovas-Massana et al., 2010). Nevertheless, a method specific for the detection of CRPA in water is lacking. Supplementing solid media with antibiotics can increase the yield in detecting resistant bacteria when their numbers are limited (Safdar et al., 2003; Murk et al., 2009). This has been used in culture methods for detecting antibiotic-resistant *P. aeruginosa* in patients, but rarely for water (Milligan et al., 2023). Currently, there is limited knowledge about the concentrations of CRPA in wastewater and surface water. In order to enable the monitoring of CRPA in wastewater and surface water, a selective method with high sensitivity is needed. The goal of this study was to establish a standardized and widely applicable method for detecting CRPA in surface water and wastewater samples.

2. Materials and methods

2.1. General approach

To establish a method for detecting CRPA by culture in wastewater and surface water samples, we started out evaluating the existing methods for the detection of *P. aeruginosa* and combining them with several antibiotics. We developed the method in a stepwise manner, by:

- Testing the phenotype and growth of well-characterized CRPA strains on four different selective agar plates supplemented with different antibiotics;
- Confirming the growth of well-characterized CRPA strains inoculated into an enrichment broth followed by culturing onto selective agar plates with different antibiotics;
- Confirming the growth of well-characterized CRPA strains spiked into surface water samples through filtration continued by culturing onto selective agar plates with different antibiotics;
- Testing the recovery of CRPA from naturally contaminated wastewater samples and evaluating the growth of background flora with direct plating by filtering wastewater samples presumptively containing CRPA followed by culturing onto selective agar plates with different antibiotics;
- Testing the recovery of CRPA from naturally contaminated wastewater samples and evaluating the growth of background flora through enrichment by filtering wastewater samples presumptively containing CRPA followed by inoculation into an enrichment broth and culturing onto selective agar plates with different antibiotics;
- Comparing method performance with antibiotics as selective agent in enrichment broth vs agar: filter surface water samples spiked with well-characterized CRPA strains, inoculate into enrichment broth with and without carbapenem for 24 and 48 h, followed by subculture onto selective agar plates with and without carbapenem.

2.2. Bacterial strains

Seven different strains of CRPA with different carbapenem-resistance genes, bla_{VIM} (3), bla_{GES-5} (1), bla_{IMP} (1), and bla_{NDM} (1), were used in this study (Table 1), hereafter named "well-characterized CRPA strains". All strains were confirmed by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS [Bruker Daltonics, Bremen, Germany]); antibiotic susceptibility was performed by VITEK-2, with additional broth microdilution (Trek Diagnostic 93 Systems, Thermo Fisher Scientific, Franklin, USA) or MIC Test Strip (Liofilchem).

2.3. Confirmation of the growth of well-characterized CRPA strains on selective agar plates

All seven well-characterized strains were used to compare four different agar plates supplemented with three different antibiotics. The selective agar plates used were *Pseudomonas* CN agar (Oxoid, Basingstoke, UK) (Slekovec et al., 2012; Weiser et al., 2014; Suzuki et al., 2013; Laine et al., 2009), modified-*Pseudomonas* agar C (M-PA-C; Becton

Table 1
Carbapenem-resistant *Pseudomonas aeruginosa* strains used in this study.

	Sequence type	Carbapenem-resistance gene	Minimum inhibitory concentration (mg/L)		
			Imipenem	Meropenem	Ceftazidime
Strain 1[a]	ST446	bla_{VIM-2}	≥16 (R)	16 (R)	16 (R)
Strain 2[b]	ST773	bla_{NDM}	>32 (R)	>32 (R)	>16 (R)
Strain 3[c]	ST111	bla_{VIM-2}	>8 (R)	4 (I)	≥32 (R)
Strain 4[d]	ST253	bla_{VIM-2}	≥16 (R)	≥16 (R)	≥16 (R)
Strain 5[e]	ST357	bla_{IMP-7}	≥16 (R)	≥16 (R)	≥32 (R)
Strain 6[e]	ST235	bla_{GES-5}	≥16 (R)	≥16 (R)	16 (R)
Strain 7[e]	ST446	None	≥16 (R)	≥16 (R)	8 (S)

[a] Previously published by van der Zee et al. (2018).
[b] From a patient that was hospitalized in Morocco, and was screened after being transferred to the Netherlands.
[c] Previously published by Pirzadian et al. (2021).
[d] Strain from a sink drain in the intensive care, previously published by Pirzadian et al. (2023).
[e] Previously published by Pelegrin et al. (2019).

Dickinson Diagnostics, Breda, The Netherlands) (de Vicente et al., 1986), CHROMagar *Pseudomonas* (CHROMagar, Paris, France) (Truong et al., 2022), and modified Z agar (mZ-agar) (Weiser et al., 2014). Antibiotics considered as supplements were imipenem (8 mg/L), meropenem (2 mg/L), and ceftazidime (8 mg/L). After 24 h of incubation at 37 °C, the colony morphologies and characteristics were observed (Fig. 1).

2.4. Confirmation of the growth of well-characterized CRPA strains in enrichment broth

Tryptone soya broth (TSB [Oxoid, Basingstoke, UK]) (Weiser et al., 2014) and asparagine proline broth (ASP [Millipore Burlington, Massachusetts, United States of America]) (Miranda et al., 2015) were used in the enrichment step of the culture method. Four different antibiotic combination supplementations through addition of antibiotic stock solutions (vancomycin 2 mg/L only, vancomycin 2 mg/L and imipenem 8 mg/L, vancomycin 2 mg/L and meropenem 2 mg/L, and vancomycin 2 mg/L and ceftazidime 8 mg/L) were used to decide which enrichment step was the optimal. The antibiotic stock solution was made using antibiotic powder from Sigma-Aldrich Co., St. Louis, MO, USA. Within this study, selective-enrichment broth was defined as broth with imipenem as selective agent for carbapenem-resistant strains and non-selective enrichment broth as broth without imipenem. Both were supplemented with vancomycin to eliminate most Gram-positive bacteria. The well-characterized strains 1–7, which were grown overnight in a standard liquid broth (i.e. brain heart infusion broth [BHI, Sigma-Aldrich Co., St. Louis, MO, USA]), 100 μL of each were inoculated into 10 mL of the test broths with and without antibiotics. After overnight incubation of the broths at 37 °C, the broths were subcultured onto selective agar plates with antibiotic stock solutions as described previously by streaking 10 μL with a sterile loop.

2.5. Growth of well-characterized CRPA strains and background flora after direct plating

To test the ability of the methods to grow the well-characterized CRPA strains from natural samples, we spiked surface water from the Nieuwe Maas river in Rotterdam, the Netherlands, with the CRPA strains. Surface water (100 mL) was spiked with approximately 10, 100, and 1000 CFU of the CRPA strains 1–7 with dilutions of fresh stocks grown overnight in BHI. The spiking volumes were estimated from concentrations of the single strains achieved after overnight growth in BHI as determined by calculating the number of colonies in decimal dilutions of overnight culture spread onto tryptone soya agar (TSA) plates. Unspiked surface water was also used to evaluate background flora. The two best agar plates with three different antibiotics based on the previous step's results were chosen along with TSA as a baseline. The spiked and unspiked water samples were directly, without pre-treatment, filtered through a 0.45 μm nitrocellulose filter (Millipore, Burlington, Massachusetts, United States of America). The filter was inoculated on the agar plates for 18–24 h at 37 °C and growth was observed along with the colony morphologies.

2.6. Growth of CRPA and background flora in naturally contaminated wastewater samples after direct plating and enrichment

For detection of CRPA in naturally contaminated water, we used a sample of the influent of the wastewater treatment plant (WWTP) located in Zwolle, the Netherlands, assuming these were contaminated with CRPA. Different volumes of the sample were used directly, without pre-treatment (10 μL, 30 μL, 100 μL, 1 mL, 3 mL, and 10 mL). The wastewater sample was filtered through a 0.45 μm pore size nitrocellulose membrane filter using active negative pressure, followed by direct inoculation onto the selective agar plates. The same selective plates as in the spiking experiment step were used. Plates were incubated for 18–24 h at 37 °C. There were two steps of the experiment. In the first experiment, 1 mL, 3 mL and 10 mL of the wastewater sample was used. The background growth on all plates was too much that background colonies could not be counted and presumptive *P. aeruginosa* colonies were difficult to count. On these plates, 15–20 presumptively *P. aeruginosa* positive colonies and 0–4 presumptively negative colonies were subcultured (from filters with 1 mL sample). In the second experiment, lower volumes of samples were used (10 μL, 30 μL, 100 μL) to achieve accountable numbers for the limit of detection. From plates inoculated with a volume equivalent to 10 μl original sample (containing <100 colonies overall), 15–32 possible *P. aeruginosa* were subcultured, in addition to 11–16 colonies presumptively representing background growth. Subsequently, species were identified using the MALDI-TOF MS. When *P. aeruginosa* was confirmed, susceptibility against carbapenems was performed by disc diffusion method using Sensi-Discs™ (Becton Dickinson, United States) and interpretation using epidemiological cut-offs (ECOFFs) as defined by EUCAST (Kahlmeter and Turnidge, 2022; European Committee on Antimicrobial Susceptibility Testing, n.d.).

The experiment was repeated by using selective enrichment (using influent samples from 3 WWTP: Lelystad, Bosscherveld and Assen, the Netherlands). One μL, 100 μL and 10 mL were filtered and enriched in broths (TSB and ASP). Vancomycin 2 mg/L (VAN) was added to all broths to decrease the growth of Gram-positive bacteria. Trimethoprim-sulfamethoxazole 1 mg/L (SXT) addition was tested to suppress growth of *Aeromonas*, which showed frequent growth in previous steps (Results section—Membrane Filtration for Wastewater Samples). The carbapenem used in this experiment was imipenem 2 mg/L (IMP) because a previous study showed more stability in laboratory conditions with higher temperatures (35 °C) than meropenem (Shahab et al., 2024a). The concentration of imipenem was lowered from 8 mg/L as used before for single strain testing to 2 mg/L in order to harmonize the environmental method with a method for human samples which was developed

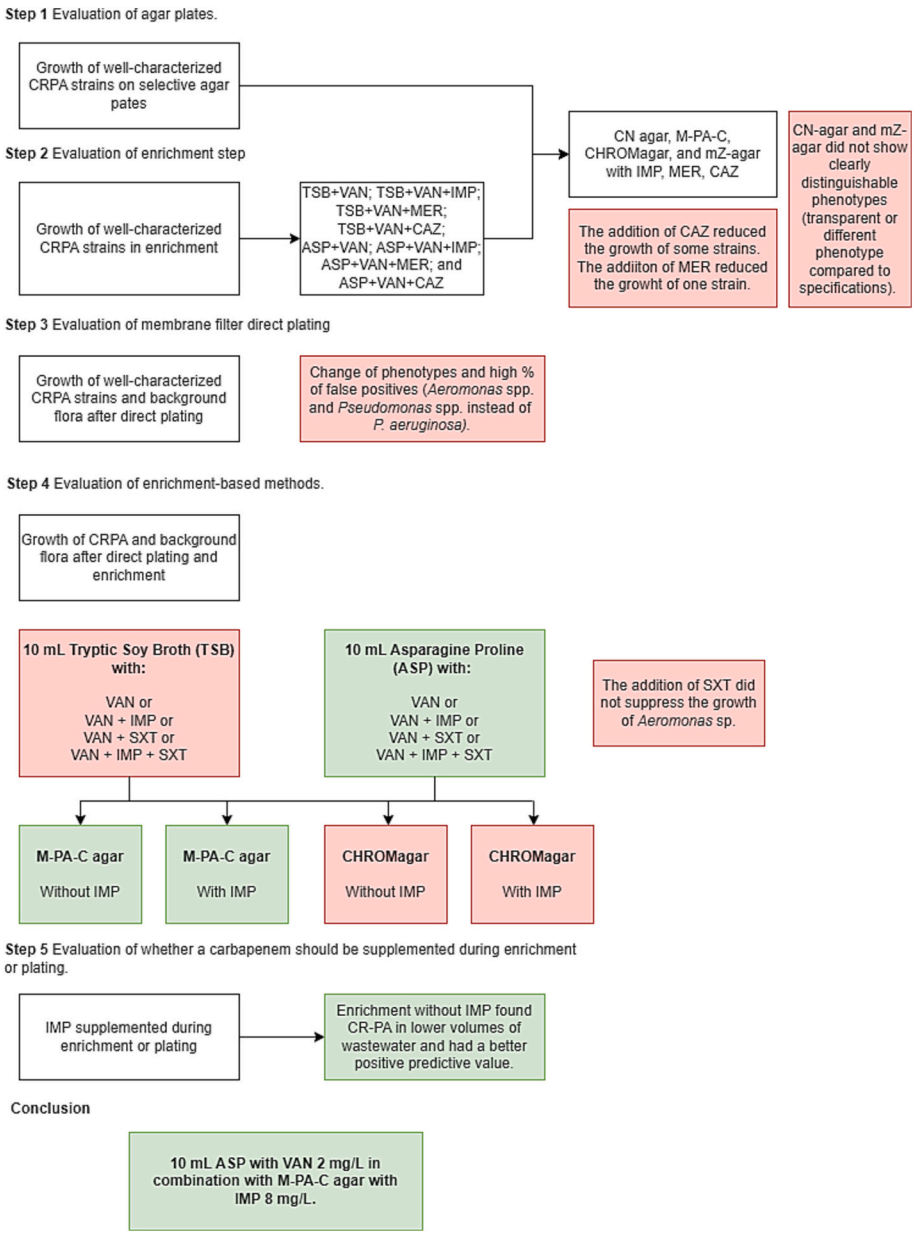

Fig. 1. Overview of the overall methods (TSB: tryptone soya broth; ASP: asparagine proline broth; CAZ: ceftazidime (8 mg/L); SXT: trimethoprim/sulfamethoxazole 1:19 (1 mg/L); IMP: imipenem (2 mg/L in broth and 8 mg/L in agar plate); MER: meropenem (2 mg/L) VAN: vancomycin (2 mg/L). Red colour: method not further pursued; green colour: method with favorable performance characteristics).

simultaneously (Shahab et al., 2024b), and the imipenem was added to broth through 10-μL antibiotic discs (SensiDiscs™, Becton Dickinson, United States) rather than through a stock solution to simplify the method for countries where antibiotic powder is more difficult to purchase than antibiotic discs. Moreover, the media preparation is easier when antibiotic discs are used. This resulted in the following combinations of broths tested in the first sample (Lelystad): TSB + VAN; TSB + VAN+IMP; TSB + VAN+SXT; TSB + VAN+SXT + IMP; ASP+VAN; ASP+VAN+IMP; ASP+VAN+SXT; and ASP+VAN+SXT + IMP. For the remaining two samples (i.e. influent from WWTP in Bosscherveld and Assen), the best performing method was used (Fig. 1). Enrichments were incubated for 18–24 h at 37 °C. After enrichment, the same plates as in the spiking experiment step were used (Fig. 1). Selective plates were inoculated with 10 μl of enrichment broth by streak plating and incubated overnight at 37 °C. From each combination of enrichment broth and plate, up to 10 isolates were confirmed by MALDI-TOF and resistance to carbapenems, including colonies with and without typical morphologies of *P. aeruginosa*.

2.7. Carbapenem supplementation in the media and length of the enrichment step

In the same wastewater samples (influent of WWTP in Lelystad, Bosscherveld and Assen that served as presumably naturally contaminated samples containing CRPA), we compared in which step carbapenem should be added; to the enrichment broth or the selective plate. To this end, the filters (volumes as above) were inoculated into enrichment broth without carbapenem and plated on selective plates with carbapenem while the filters inoculated into selective-enrichment broth with carbapenem were plated on plates without carbapenem. Thus, the

following broth combinations were used for influent from Lelystad in addition to the enrichment broths used in 2.6: TSB + VAN; TSB + VAN+SXT; ASP+VAN; ASP+VAN+SXT. For the two remaining samples (Bosscherveld and Assen), ASP+VAN was used as selective enrichment. Enrichments of these broths were plated on selective plates (M-PA-C and CHROMagar) with imipenem. Selective plates were inoculated with 10 μl of enrichment broth by streak plating. From each combination of enrichment broth and plate, up to 10 isolates were analysed by MALDI-TOF for species identification and disc diffusion for resistance to carbapenems.

To determine the limit of detection of the two best methods and the optimal incubation time for the enrichment step, we compared different incubation times using the best broth and the best plate from the previous comparisons. These were: enrichment in ASP + VAN followed by plating on M-PA-C supplemented with imipenem, and enrichment in ASP + VAN + IMP followed by plating on M-PAC without imipenem supplementation. Serially diluted CRPA strains 1–7 directly inoculated into the enrichment broth were used. The amount of well-characterized CRPA strains spiked was determined by counting overnight cultures used for inoculating the enrichment broths onto TSA. Subculture of the broth was performed after 18–24 and 48 h of incubation. Different steps of carbapenem supplementation were also applied. The growth on the selective agar plates was observed.

2.8. Data analysis

True positives were defined as the number of colonies showing morphologies according to *Pseudomonas* on the agar plates and confirmed first as *P. aeruginosa*, second as CRPA. Species identification was performed using the MALDI-TOF MS and resistance against carbapenems was confirmed by performing disc diffusion using Sensi-Discs™ (Kahlmeter and Turnidge, 2022; European Committee on Antimicrobial Susceptibility Testing, n.d.). CRPA was defined as imipenem-resistant and/or meropenem-resistant *P. aeruginosa* based on the antibiotic susceptibility test results. True negatives were the number of colonies showing morphologies atypical for *P. aeruginosa* on the agar plates and confirmed as species other than *P. aeruginosa*.

Sensitivity was measured as the number of true positives of CRPA colonies divided by the number of all colonies (with and without the presumptively positive phenotype) confirmed as *P. aeruginosa* or CRPA. Specificity was defined as the number of true negatives divided by the number of all isolates (with and without the presumptively positive phenotype) confirmed as non—*P. aeruginosa* or CRPA. Positive predictive value (PPV) was the number of true positives divided by the total number of colonies tested. Negative predictive value was the number of true negatives divided by the number of isolates showing morphologies of non-*P. aeruginosa*. The agreement was defined as the sum of the true positives and true negatives divided by the total number of isolates tested for confirmation of the identification.

3. Results

3.1. Comparison of the growth of well-characterized CRPA strains on selective agar plates

Fig. 2 shows the colony morphologies on different agar plates supplemented with imipenem. For other antibiotics, the morphologies showed similar colony morphologies. All strains grew well with correct morphologies on the M-PA-C agar with three different antibiotics (Supplementary Table 1). A variety of morphologies on the M-PA-C agar also agreed with the manufacturer's descriptions (Supplementary Fig. 1). On CHROMagar, all strains grew with correct morphologies despite of three strains showing reduced growth when using ceftazidime. On the CN agar, Strain 2 and Strain 3 grew less when using imipenem while Strain 6 and Strain 7 grew less when using ceftazidime (Supplementary Table 1). Despite growing well, Strain 1 and Strain 5 did not produce pigment on CN agar (Supplementary Fig. 2). On the mZ-agar, three strains grew less when using meropenem and did not grow when using imipenem, another strain did not grow when using meropenem, and Strain 7 did not grow when meropenem and ceftazidime were used. CRPA growth on mZ-agar created transparent colonies that were difficult to be read. From this experiment, M-PA-C and CHROMagar were chosen as the best agar plates for the detection of CRPA.

3.2. Confirmation of the growth of well-characterized CRPA strains in enrichment broth

After confirmation of the growth of well-characterized CRPA strains following enrichment in different combinations of media and carbapenem supplements (Supplementary Table 1), M-PA-C and CHROMagar were again the best agar plates for CRPA as all strains could grow well except Strain 6, which did not grow on M-PA-C and CHROMagar with ceftazidime after enrichment with ASP with ceftazidime. Three strains did not grow well on mZ-agar supplemented with imipenem or meropenem while another strain did not grow well on mZ-agar supplemented with ceftazidime.

3.3. Growth of well-characterized CRPA strains spiked in surface water samples with direct plating

When unspiked surface water was used (100 mL), no growth was found on M-PA-C with imipenem and M-PA-C with meropenem. Limited growth was found on the other media: on M-PA-C with ceftazidime, CHROMagar with imipenem, CHROMagar with meropenem, and CHROMagar with ceftazidime, 8, 2, 39, and 9 colonies grew, respectively. None of them were CRPA colonies. >100 colonies grew on TSA. Strains 1–5 grew well on all plates with different antibiotics (Supplementary Table 2). Strain 6 grew less on M-PA-C with ceftazidime and CHROMagar with imipenem. Strain 6 and 7 did not grow on CHROMagar with ceftazidime. In most strains, the use of filters led to phenotypic changes: faded pigment of the colonies on CHROMagar plates, yielding transparent colonies with a hint of brown or grey instead of blue or greenish-blue (Fig. 3, compared to Fig. 2). Colonies of well-characterized CRPA strains on M-PA-C plates were pink (correct) and some brownish pink (Strain 2 and Strain 4) or yellowish pink (some colonies of Strain 6).

3.4. Recovery of CRPA and background growth in wastewater samples after direct plating

For testing the recovery of CRPA from naturally contaminated samples, wastewater samples presumptively containing CRPA and background flora were used. Low volumes of wastewater (10, 30 and 100 μL) yielded a limited number of *P. aeruginosa* on all plates tested among the presumptively positive samples. In addition, a high amount of background flora was found on all combinations of plates tested. In a volume equivalent to 1 mL of the original sample, we found 20 different presumptive *P. aeruginosa* colonies on M-PA-C with meropenem and imipenem, of which 3 were confirmed as CRPA and 1 as carbapenem-susceptible *P. aeruginosa* on M-PA-C with meropenem and 1 as CRPA on M-PA-C with imipenem. On M-PA-C with ceftazidime, CHROMagar with imipenem, and CHROMagar with ceftazidime, we found 40, 31, and 35 different presumptive *P. aeruginosa* colonies, of which none was confirmed as CRPA. On CHROMagar with meropenem, one colony of CRPA was found, but it was not the colour that was suspected as *P. aeruginosa*. As in the previous experiment, the CRPA colonies on M-PA-C agar were brownish and yellowish pink, both with imipenem and with meropenem addition (Fig. 4). Blue colonies with a halo on CHROMagar were false positive (Fig. 4), both with meropenem and with imipenem addition. Fig. 5 describes the other species found and Fig. 6 describes the number of *Pseudomonas* spp. among all suspected colonies found on different agar plates with different antibiotics.

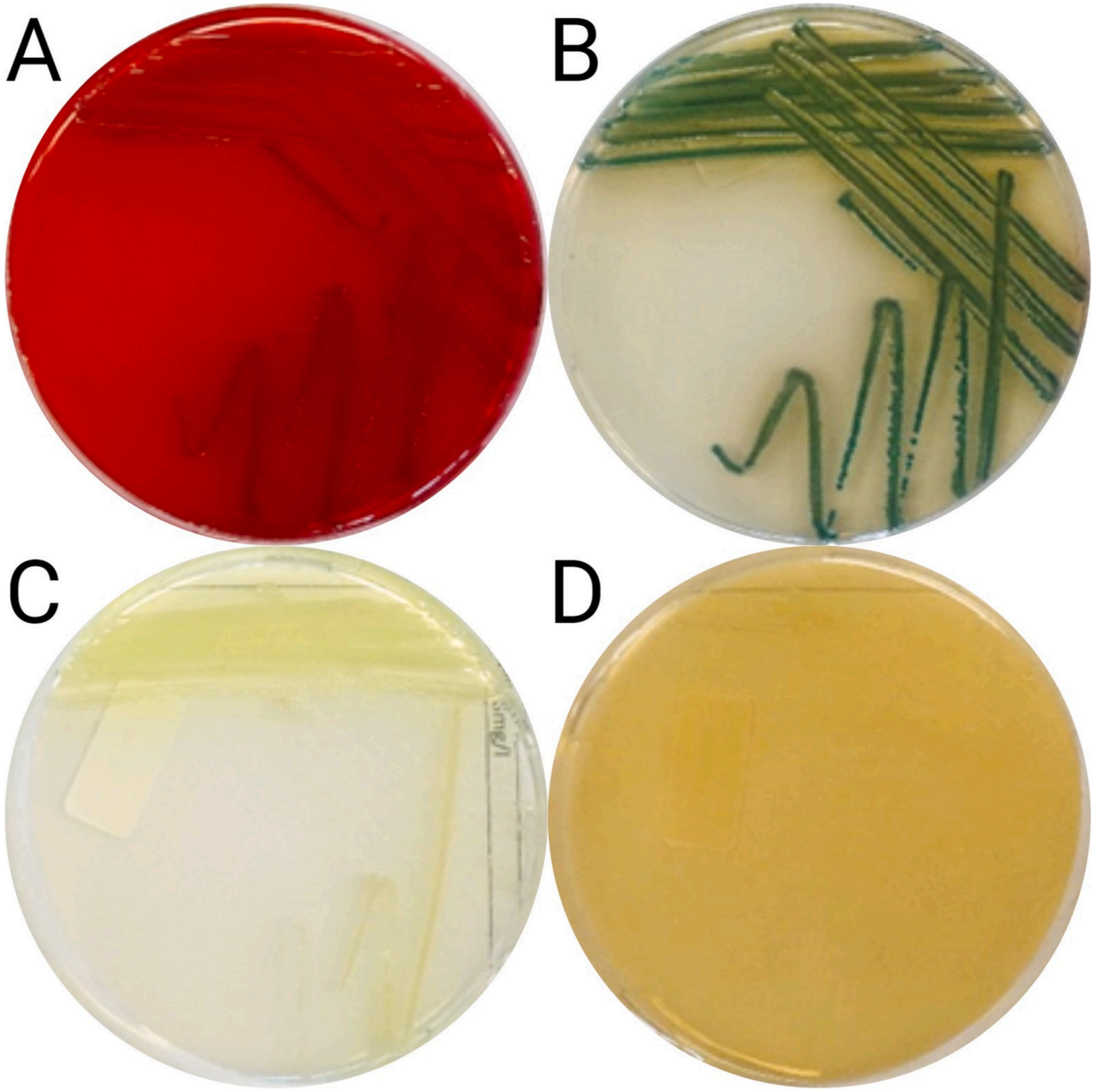

Fig. 2. Morphologies of Strain 4 on different agar plates with imipenem (A: M-PA-C; B: CHROMagar; C: CN agar; D: mZ-agar).

3.5. Recovery of CRPA and background growth in wastewater samples with enrichment with and without carbapenem supplementation

For testing recovery of CRPA strains from naturally contaminated samples with detection based on enrichment, wastewater samples presumptively containing CRPA and extensive background flora were used. In this experiment, imipenem was chosen over meropenem because a previous study showed meropenem was less stable when at higher temperature (35 °C) which was used for the incubation during the culture (Shahab et al., 2024a; Fawaz et al., 2019). In addition, cotrimoxazole supplementation during enrichment was tested for its ability to inhibit the growth of *Aeromonas* sp. that were often found in the previous experiments. Table 2 shows the method performance characteristics of CRPA detection based on different enrichment media with and without imipenem and cotrimoxazole, followed by subculturing onto selective agar plates with and without imipenem. The most critical method parameter was the PPV, as a considerable proportion of presumptive CRPA turned out as either non—*P. aeruginosa*, or as *P. aeruginosa* non-resistant to carbapenem antibiotic. The addition of cotrimoxazole did not suppress the growth of *Aeromonas* sp., in fact, the PPV was generally lower when cotrimoxazole was used in the enrichment (Supplementary Table 2). From samples subcultured onto M-PA-C and CHROMagar with and without imipenem, CRPA growth was always found in the same or lower volume of sample on M-PA-C agar plates as compared to CHROMagar (Table 3). Also, mostly, M-PA-C had a higher PPV (e.g. 80 % after enrichment in ASP) as compared to CHROMagar (e.g. 30 % after enrichment in ASP). There was no clear trend in PPV or detection of CRPA at lower sample volumes between TSB and ASP as enrichment broths. Addition of imipenem in the agar plate had higher sensitivity than the addition in the broth, in terms of detection of CRPA at lower volumes of sample, although the PPV for detection of CRPA with addition of imipenem to the plate was lower than addition to the broth (e.g. 30 % for ASP+VAN continued by subculturing onto CHROMagar+IMP compared to 100 % ASP+VAN+IMP continued by

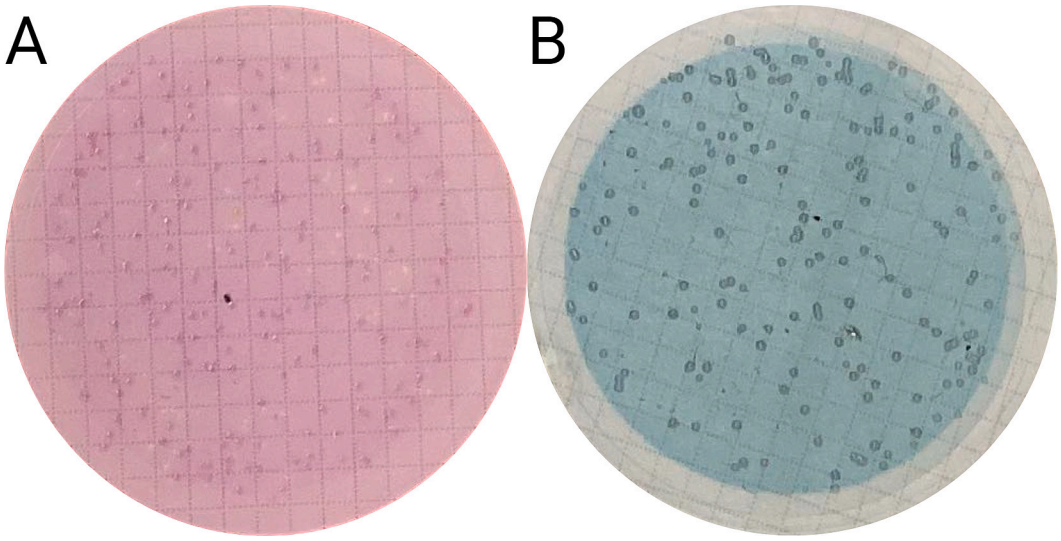

Fig. 3. Surface water (100 mL) spiked with Strain 5 (100 CFU) on M-PA-C (A) and CHROMagar (B) with imipenem.

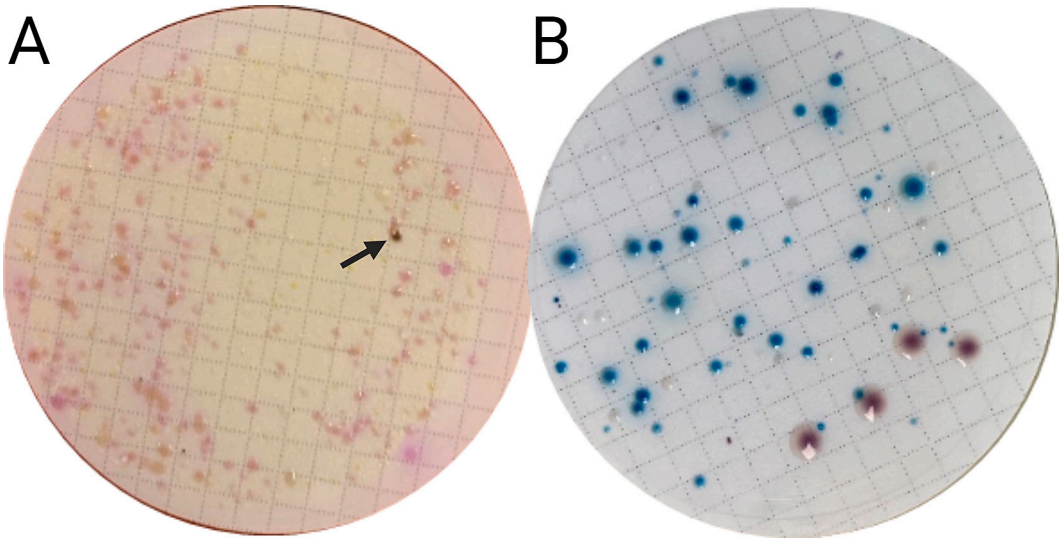

Fig. 4. Colonies on M-PA-C (A) and CHROMagar (B) with imipenem using 3 mL of wastewater sample. On this particular M-PA-C plate, there was 1 carbapenem-resistant *Pseudomonas aeruginosa* (CRPA; marked with an arrow). On CHROMagar, on this particular plate, there were neither CRPA nor *P. aeruginosa* found. All blue colonies on CHROMagar are other species.

subculturing onto CHROMagar). Therefore, one follow-up experiment was executed, using M-PA-C, to confirm that carbapenem supplementation in the plates would be superior to supplementation during enrichment with respect to the limit of detection. Table 3 shows that when comparing addition of imipenem in asparagine proline broth during enrichment versus addition to the M-PA-C agar plate during plating, more CRPA strains were found (32 colonies of 44 suspected colonies) from the addition of imipenem in the agar plate. Supplementing imipenem to the broth caused more false positive colonies (non-*P. aeruginosa* species), but all *P. aeruginosa* found were carbapenem-resistant. Again, the combination of nonselective enrichment with supplementation of the agar plates with imipenem led to more sensitive detection of CRPA (in terms of positive findings in lower sample volumes). Moreover, addition of imipenem to selective agar plates instead of broth resulted in a higher PPV (73 % vs 32 %).

The lower limit of detection (Table 4) as determined from well-characterized CRPA strains spiked into enrichment broth confirmed that supplementation of imipenem in the enrichment broth led to the detection of known CRPA at higher concentrations. A longer incubation period of the enrichment step (48 h instead of 24 h) resulted only in additional colonies of Strain 6, which changed the lower limit of detection from 7.20×10^2 to 7.20 CFU.

4. Discussion

This study provides a standardized culture-based method for quantification of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) in both surface water and wastewater. In developing such method, various selective media, enrichment steps, antibiotic supplementations, and filtration were systematically evaluated. The best culture method for detecting CRPA in surface water and wastewater is by filtering the water, followed by inoculating into selective broth (i.e. asparagine proline broth with vancomycin), continued by subculturing to selective agar plate (i.e. M-PA-C) containing imipenem.

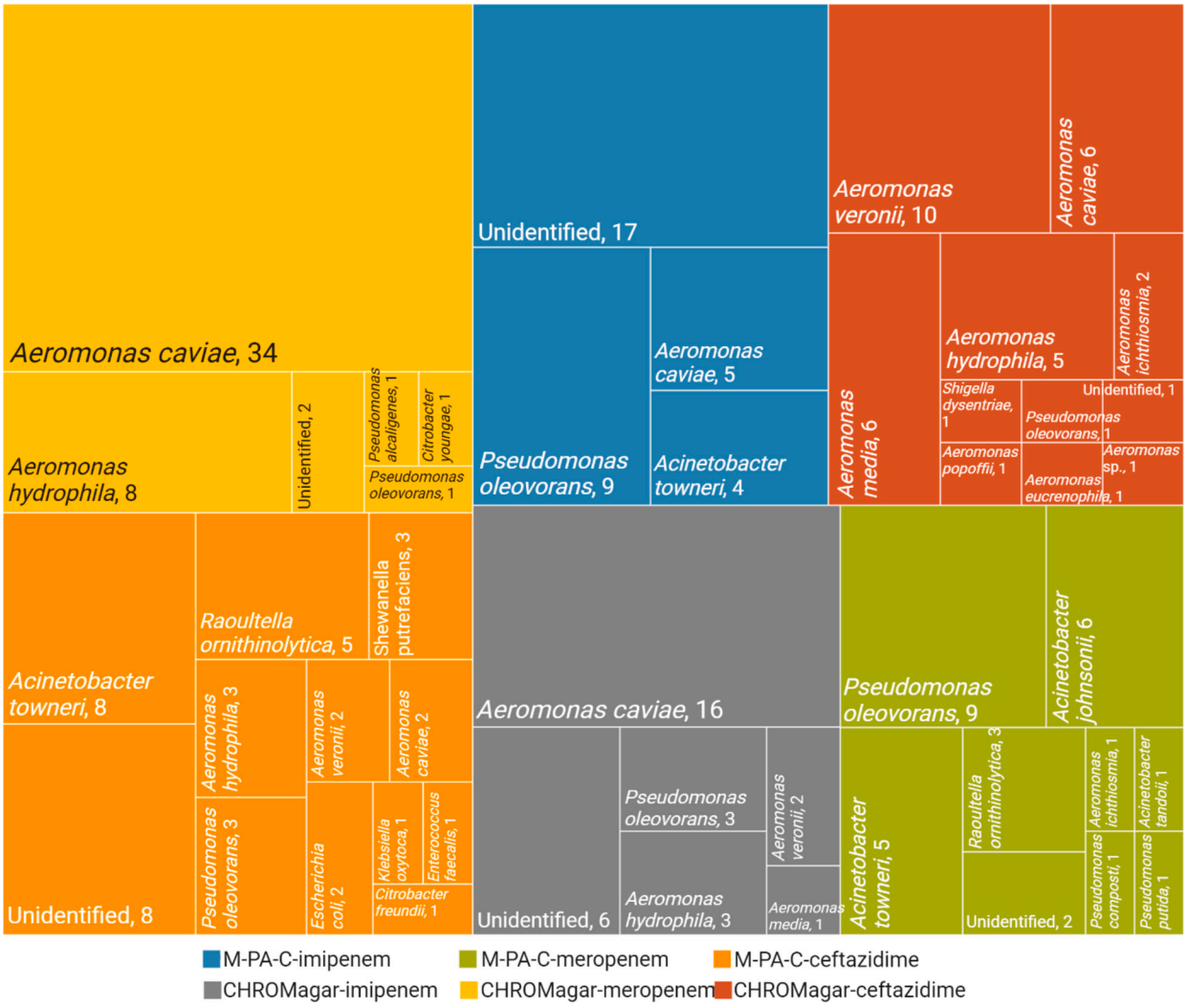

Fig. 5. False positive species identification during direct plating of wastewater on M-PA-C and CHROMagar supplemented with antibiotics.

4.1. Comparison of selective media supplemented with antibiotics for detection of CRPA

The culture media compared in this study have been chosen from media that have previously been used (Slekovec et al., 2012; Weiser et al., 2014; Suzuki et al., 2013; Laine et al., 2009; de Vicente et al., 1986; Truong et al., 2022; Miranda et al., 2015). Both solid and liquid culture media were chosen based on the availability and previous performance reported.

The first step was to choose the selective agar plates for CRPA in which well-characterized strains showed clear and phenotypically similar growth. In this step, four different *Pseudomonas* selective agar plates were compared. CN agar exhibited inconsistent pigmentation, and mZ agar had poor colony visibility and was not successful in growing well-characterized strains, and both were negatively affected by antibiotic supplementation as observed in a previous study (Weiser et al., 2014). In contrast, M-PA-C and CHROMagar consistently supported CRPA growth, with and without enrichment step, and displayed colony morphologies as described by the manufacturers.

Previously, ceftazidime (2 mg/L) has been used as a supplement in culture media for screening for bla_{VIM}-positive *P. aeruginosa* (Voor In 't Holt et al., 2018). In our study, ceftazidime 8 mg/L in M-PA-C and CHROMagar inhibited the growth of one respectively two well-characterized CRPA strains in this study. In a detection method, the selective agar plate must be sensitive enough for CRPA and at the same time be practical in differentiating *P. aeruginosa* from other bacteria. Therefore, the use of ceftazidime 8 mg/L in the media to detect CRPA is not recommended.

4.2. Filtration and direct plating vs enrichment step

Filtration is recommended for sample pretreatment as it allows for the concentration of bacteria from large volumes of water.

While the use of filters for direct plating onto selective agar would be beneficial for quantitative determinations, it led to a high number of false positive isolates resulting in a low PPV. For instance, blue colonies on CHROMagar plate with imipenem only consisted of species other than *P. aeruginosa*, predominantly *Aeromonas caviae*. In addition, filter inoculation can alter the morphology of well-characterized CRPA colonies on selective agar, hindering accurate identification.

To improve the specificity of detection and reduce false positives, an enrichment step using selective broth is recommended. Enrichment still allows for the use of filtration; such that large volumes of water can be analysed. Nevertheless, the disadvantage is that it adds at least 24 h to the process. Furthermore, enrichments require a most-probable-number approach for quantification, in turn requiring analysing a large number of replicates per single sample, increasing the overall labor requirements. When incubated for an additional 24 h (48 h of incubation in the enrichment step), only a few additional target colonies grew, suggesting that a longer enrichment period may not significantly

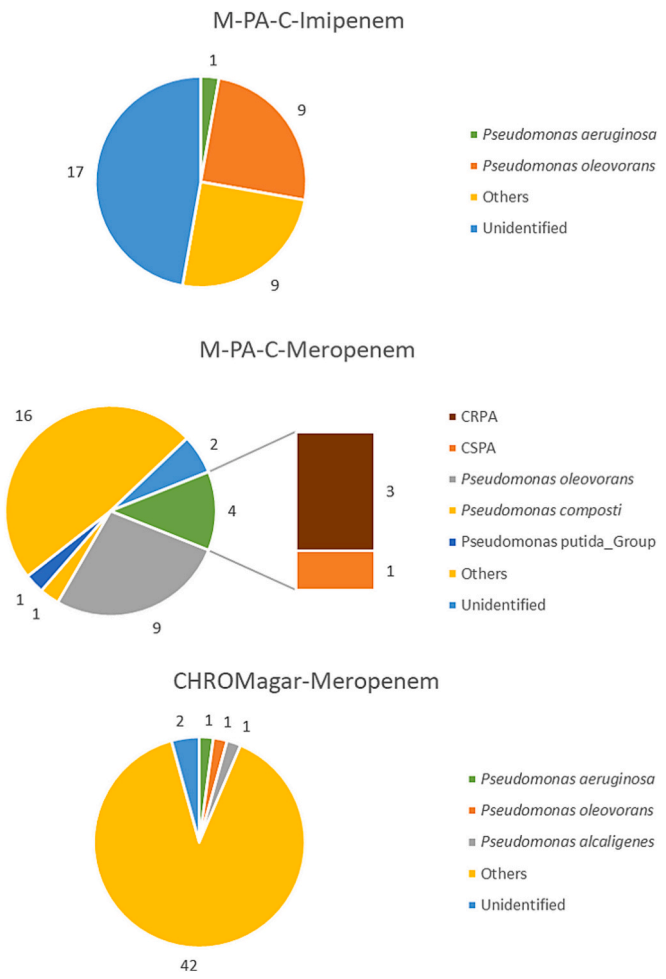

Fig. 6. Proportion of *Pseudomonas aeruginosa* and *Pseudomonas* spp. compared to all suspected colonies found during direct plating of wastewater on M-PA-C and CHROMagar supplemented with antibiotics. CRPA: carbapenem-resistant *Pseudomonas aeruginosa*; CSPA: carbapenem-susceptible *Pseudomonas aeruginosa*.

improve detection. This long process of enrichment might therefore only be useful when aiming for detecting a very small number of CRPA in a sample.

The requirement of an enrichment step remains a disadvantage of the method provided here, given the extra workload and time requirement of the enrichment step. Future analyses could be directed at evaluating additional media used for drinking water and recreational water, such as Rapid *P. aeruginosa* agar, to potentially streamline the detection process and reduce the need for extended enrichment.

4.3. Carbapenem supplementation as selective agent in liquid vs solid media

In addition, for the enrichment procedure, carbapenem supplementation in the broth and the agar plates was compared. Addition of imipenem in the broth may eliminate some of the CRPA strains, producing lower sensitivity and increasing the lower limit of detection (Tables 3–4). Moreover, more colonies suspected as CRPA were false positive when imipenem was added to the broth resulting in lower PPV (32 % vs 73 %).

4.4. Study limitations

This study has some limitations. First, although vancomycin might alter the number of CRPA in the sample, an enrichment step without vancomycin was not compared since this method was established in a joint One Health effort in which also methods for analysis of CRPA in human feces and hospital environmental samples were established (https://classic.clinicaltrials.gov/show/NCT05282082, n.d.). For these, usage of vancomycin was necessary to limit the growth of Gram-positive bacteria. Furthermore, due to harmonization with a method for human diagnostics, the evaluation of different incubation temperatures was restricted. This newly developed method for diagnosis of CRPA in human feces and hospital environmental samples (citation) was based on culturing at 36 °C, chosen to enable execution of this protocol also in resource-limited countries, where only a single stove is available in clinical diagnostic laboratories, operating at 36 °C. Higher incubation temperatures (42 °C) have been suggested in order to reduce growth of environmental background flora, and might therefore improve the PPV (Schreiber et al., 2021). The standard incubation temperature chosen here allows this method to be widely applicable and in line with a One Health approach where clinical and environmental samples can be incubated in the same incubator.

Second, only seven well-characterized CRPA strains were used. Nevertheless, those seven strains have different genotypes and carbapenem-resistance genes and they came both from Indonesia and the Netherlands. Third, wastewater and surface water samples tested in this study were from the Netherlands. Wastewater from other parts of the world may contain different background flora.

4.5. Outlook

While environmental surveillance of antibiotic-resistant bacteria is called for internationally, the implementation is still hindered by a lack of suitable methods (Milligan et al., 2023; Schreiber et al., 2021). For CRPA in particular, insight in transmission through environmental surfaces in healthcare institutions and through wastewater and surface water is urgently needed, given that *Pseudomonas* can well survive in water (Lewenza et al., 2018). In order to determine the role of environmental transmission and attribute human carriage across different transmission routes, insight in the genetic relatedness between resistant bacteria recovered from environmental compartments that are representative of particular potential transmission routes and clinical isolates is required. However, this is only possible with culture-based methods. Thus, the method established here can both help environmental surveillance, and dedicated studies into the roles of waterborne transmission of CRPA. By providing a robust cultural method, it can improve the ability to isolate and characterize CRPA strains from diverse environmental sources. This will enable researchers to gain valuable insights into the epidemiology of CRPA and develop effective strategies to mitigate its spread.

5. Conclusion

This study compared several approaches to detect CRPA in surface water and wastewater. Strategies described by previous studies were compared, adapted, and combined to reach the optimal method. Ideally, not only the method shall capture various strains of CRPA, but it must also exclude background flora found in water. Therefore, the use of selective media supplemented by antibiotics is necessary. The following considerations are suggested in culturing CRPA from water samples:

1. With respect to solid media, the use of M-PA-C agar plates supplemented with imipenem is recommended to limit the growth of other bacteria and carbapenem-susceptible *P. aeruginosa*.
2. An enrichment step is recommended to eliminate the background flora of the water samples. Enrichment is superior to direct plating with respect to the positive predictive value. The high amount of background growth during direct plating makes direct plating practically infeasible.

Table 2
Method performance characteristics of determination of CRPA with enrichment using different antibiotics in detecting CRPA in wastewater samples.

Enrichment step	Selective agar plate	n of colonies tested	*Pseudomonas aeruginosa*						CRPA					
			SENS	SPEC	PPV	NPV	Agree-ment	Vol (mL)	SENS	SPEC	PPV	NPV	Agree-ment	Vol (mL)
TSB + VAN	M-PA-C + IMP	10	100	*	100	*	100	10^{-1}	100	*	100	*	100	10^{-1}
	CHROMagar + IMP	10	100	*	100	*	100	10	100	0	50	*	50	10
ASP + VAN	M-PA-C + IMP	10	100	*	100	*	100	10	100	0	80	*	80	10
	CHROMagar + IMP	10	100	*	100	*	100	10	100	0	30	*	30	10
TSB + VAN + IMP	M-PA-C	5	**	100	**	100	100	**	**	100	**	100	100	**
	CHROMagar	5	**	0	0	*	0	**	**	0	0	*	0	**
ASP + VAN + IMP	M-PA-C	5	100	*	100	*	100	10	100	*	100	*	100	10
	CHROMagar	5	100	*	100	*	100	10	100	*	100	*	100	10
TSB + VAN + IMP	M-PA-C + IMP	5	**	100	**	100	100	**	**	100	**	100	100	**
	CHROMagar + IMP	10	100	0	40	*	40	10	100	0	40	*	40	10
ASP + VAN + IMP	M-PA-C + IMP	5	100	*	100	*	100	10	100	*	100	*	100	10
	CHROMagar + IMP	5	100	*	100	*	100	10	100	*	100	*	100	10

*No negative result.
**No positive result.
Red colour shows PPV and agreement <100 % (the darker the shade, the lower PPV or agreement).
n of colonies tested: number of colonies tested.
SENS: sensitivity (%).
SPEC: specificity (%).
PPV: positive predictive value (%).
NPV: negative predictive value (%).
Vol: first lowest volume positive (mL).
TSB: tryptone soya broth.
ASP: asparagine proline broth.
TSB + VAN: tryptone soya broth with vancomycin (2 mg/L).
ASP + VAN: asparagine proline broth with vancomycin (2 mg/L).
TSB + VAN + IMP: tryptone soya broth with vancomycin (2 mg/L) and imipenem (2 mg/L).
ASP+ VAN + IMP: asparagine proline broth with vancomycin (2 mg/L) and imipenem (2 mg/L).
M-PA-C + IMP: modified-*Pseudomonas* agar C with imipenem (8 mg/L).
CHROMagar + IMP: CHROMagar with imipenem (8 mg/L).

Table 3
Comparison of accuracy and lowest volume of wastewater samples in which *Pseudomonas aeruginosa* and carbapenem-resistant *Pseudomonas aeruginosa* strains were detected.

	n colonies tested	*Pseudomonas aeruginosa*								CRPA							
		SENS	SPEC	PPV	NPV	Agreement	Vol (mL)			SENS	SPEC	PPV	NPV	Agreement	Vol (mL)		
							Exp 1	Exp 2	Exp 3						Exp 1	Exp 2	Exp 3
ASP+ VAN ➔ M-PA-C + IMP	44	100	[a]	100	[a]	100	10	10^{-3}	10^{-3}	100	0	73	[a]	73	10	10^{-3}	10^{-3}
ASP + VAN + IMP ➔ M-PA-C	31	100	0	32	[a]	32	10	10	>10	100	0	32	[a]	32	10	10	>10

ASP + VAN ➔ M-PA-C + IMP: Inoculation of membrane filter to asparagine proline broth with vancomycin (2 mg/L) followed by subculturing onto M-PA-C agar with imipenem (8 mg/L).
ASP + VAN + IMP ➔ M-PA-C: Inoculation of membrane filter to asparagine proline broth with vancomycin (2 mg/L) and imipenem followed by subculturing onto M-PA-C agar.
n colonies tested: number of colonies tested.
SENS: sensitivity (%).
SPEC: specificity (%).
PPV: positive predictive value (%).
NPV: negative predictive value (%).
Vol: first lowest volume positive (mL).
[a] No negative results.

Table 4
Lower limit of detection of two methods with different imipenem supplementation step to detect CRPA in the sample of surface water spiked with well-characterized CRPA.

	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7
ASP +VAN > M-PA-C + IMP	3.91 CFU	<2.03 CFU	$<1.11 \times 10^1$ CFU	<2.04 CFU	$<2.09 \times 10^1$ CFU	7.20×10^1 CFU	<1.28 CFU
ASP + VAN +IMP > M-PA-C	$>3.91 \times 10^3$ CFU	<2.03 CFU	$>1.11 \times 10^4$ CFU	$>2.04 \times 10^3$ CFU	2.09 CFU	7.20×10^2 CFU[a]	<1.28 CFU

CFU: colony forming unit.
ASP + VAN > M-PA-C + IMP: Inoculation of membrane filter to asparagine proline broth with vancomycin (2 mg/L) continued by subculturing onto M-PA-C agar with imipenem (8 mg/L).
ASP + VAN + IMP > M-PA-C: Inoculation of membrane filter to asparagine proline broth with vancomycin (2 mg/L) and imipenem (2 mg/L) continued by subculturing onto M-PA-C agar.
[a] Decreased to 7.20 CFU when the enrichment period was 48 h.

3. When an enrichment step is applied, addition of imipenem (2 mg/L) to the broth is not recommended because it can eliminate some of the CRPA isolates and increase the lower limit of detection.
4. Imipenem (8 mg/L) as the selective agent added to an agar plate showed lower limits of detection as compared to supplementation of the enrichment broth. Addition of ceftazidime (8 mg/L) instead of carbapenem might inhibit some CRPA from growing.
5. Filtration followed by enrichment allows a large volume of water to be analysed.

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CRediT authorship contribution statement

Selvi N. Shahab: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Anneloes van Veen:** Writing – review & editing, Project administration, Conceptualization. **Merel A. Kemper:** Writing – review & editing, Validation, Resources, Investigation. **Amber Rijfkogel:** Writing – review & editing, Validation. **Margreet C. Vos:** Writing – review & editing, Supervision. **Anis Karuniawati:** Writing – review & editing, Supervision, Funding acquisition. **Juliëtte A. Severin:** Writing – review & editing, Supervision, Funding acquisition. **Heike Schmitt:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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