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Antibiotic resistance trends among *Vibrio vulnificus* and *Vibrio parahaemolyticus* isolated from the Chesapeake Bay, Maryland: a longitudinal study

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ABSTRACT Antibiotics are often used to treat severe *Vibrio* infections, with third-generation cephalosporins and tetracyclines combined or fluoroquinolones alone being recommended by the US Centers for Disease Control and Prevention. Increases in antibiotic resistance of both environmental and clinical vibrios are of concern; however, limited longitudinal data have been generated among environmental isolates to inform how resistance patterns may be changing over time. Hence, we evaluated long-term trends in antibiotic resistance of vibrios isolated from Chesapeake Bay waters (Maryland) across two 3-year sampling periods (2009–2012 and 2019–2022). *Vibrio parahaemolyticus* (n = 134) and *Vibrio vulnificus* (n = 94) to*x*R-confirmed isolates were randomly selected from both sampling periods and tested for antimicrobial susceptibility against eight antibiotics using the Kirby-Bauer disk diffusion method. A high percentage (94%–96%) of *V. parahaemolyticus* isolates from both sampling periods were resistant to ampicillin and only 2%–6% of these isolates expressed intermediate resistance or resistance to third-generation cephalosporins, amikacin, tetracycline, and trimethoprim-sulfamethoxazole. Even lower percentages of resistant *V. vulnificus* isolates were observed and those were mostly recovered from 2009 to 2012, however, the presence of multiple virulence factors was observed. The frequency of multi-drug resistance was relatively low (6%–8%) but included resistance against antibiotics used to treat severe vibriosis in adults and children. All isolates were susceptible to ciprofloxacin, a fluoroquinolone, indicating its sustained efficacy as a first-line agent in the treatment of severe vibriosis. Overall, our data indicate that antibiotic resistance patterns among *V. parahaemolyticus* and *V. vulnificus* recovered from the lower Chesapeake Bay have remained relatively stable since 2009.

IMPORTANCE *Vibrio* spp. have historically been susceptible to most clinically relevant antibiotics; however, resistance and intermediate-resistance have been increasingly recorded in both environmental and clinical isolates. Our data showed that while the percentage of multi-drug resistance and resistance to antibiotics was relatively low and stable across time, some *Vibrio* isolates displayed resistance and intermediate resistance to antibiotics typically used to treat severe vibriosis (e.g., third-generation cephalosporins, tetracyclines, sulfamethoxazole-trimethoprim, and aminoglycosides). Also, given the high case fatality rates observed with *Vibrio vulnificus* infections, the presence of multiple virulence factors in the tested isolates is concerning. Nevertheless, the continued susceptibility of all tested isolates against ciprofloxacin, a fluoroquinolone, is indicative of its use as an effective first-line treatment of severe *Vibrio* spp. infections stemming from exposure to Chesapeake Bay waters or contaminated seafood ingestion.

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KEYWORDS vibrios, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, antibiotic resistance, Chesapeake Bay, vibriosis

Non-cholera *Vibrio* spp., primarily pathogenic *Vibrio vulnificus* and *Vibrio parahaemolyticus*, are responsible for an estimated 80,000 illnesses and 100 fatalities each year in the United States (1). These Gram-negative bacteria are causative agents of gastroenteritis, wound infections, and primary septicemia associated with seafood consumption and brackish or ocean water exposure (2–5). Although more than half of all cases of *Vibrio* illness (vibriosis) in the U.S. can be attributed to the ingestion of *V. parahaemolyticus* contaminated raw or undercooked shellfish (5–7), severe and fatal cases are more frequent following *V. vulnificus* infection from water exposure (8–11). Individuals with underlying medical conditions, such as diabetes, liver disease, and immunocompromised systems, are at a greater risk of acquiring severe vibriosis (3, 5, 10). Most infections occur during the summer months, when water temperatures are highest (6, 12–15), with noted increasing annual incidence rates of *Vibrio* spp. illness during the last few decades (16–18).

While the majority of vibriosis cases are mild and self-limiting and do not require clinical treatment, antibiotics are used to treat more severe infections and prompt administration, usually within 24–48 h. of exposure, significantly improves case-fatality rates of *V. vulnificus* associated wound infections (10, 12). Tetracycline (TE) and third-generation cephalosporins [e.g., cefotaxime (CTX), ceftazidime (CAZ)] have been traditionally used in the treatment of both primary septicemia and wound infections caused by *V. vulnificus* and to a lesser degree *V. parahaemolyticus* (10, 18, 19). According to Tang et al. (19), cephalosporins and TEs (including doxycycline) combined, rather than single drug regimens, may be more effective at treating severe *V. vulnificus* infections (18). In addition, newer fluoroquinolones [e.g., ciprofloxacin (CIP) and levofloxacin], which display greater potency and a broader spectrum of antimicrobial activity and effectiveness, have been suggested as an alternative single-agent treatment for severe vibriosis (18, 19). Indeed, results from a long-term analysis of the U.S. Centers for Disease Control and Prevention's (CDC) *Vibrio* surveillance data, showed that mortality rates for *V. vulnificus* were significantly lower in patients taking either a fluoroquinolone only or a TE combined with a third-generation cephalosporin (18). Conversely, among children for whom doxycycline and fluoroquinolones are contraindicated, trimethoprim-sulfamethoxazole (SXT) plus an aminoglycoside [e.g., amikacin (AK), gentamicin] are recommended instead (20).

Although *Vibrio* spp. are generally considered susceptible to common-use antibiotics (10, 19), resistance and intermediate-resistance have been increasingly recorded in both environmental (from seafood and seawater samples) and clinical isolates (21–32). Notably, a high percentage of resistance to penicillin and ampicillin (AMP) has been observed in both *V. parahaemolyticus* and *V. vulnificus* (21, 22), and previous studies have suggested that these antibiotics may no longer be effective as a single antibiotic treatment for vibriosis (18, 33). Excessive use of antibiotics in humans, aquaculture, and agricultural settings (e.g., poultry farms) plays a major role in the selection of antibiotic resistance through horizontal gene transfer among many bacterial genera, including *Vibrio* spp. (33, 34). The persistence of antibiotics in aquatic environments, which function as critical reservoirs, can promote the evolution and transfer of antibiotic resistance genes among bacterial species and subsequently across the food chain (35).

Shaw et al. in 2014 completed the most recent antimicrobial susceptibility study of *V. parahaemolyticus* and *V. vulnificus* environmental isolates recovered from Chesapeake Bay waters in Maryland, and included strains collected during the summer of 2009 (21). Results indicated that while antibiotics used to treat adult vibriosis were fully effective at suppressing the growth of recovered isolates, pediatric-use antibiotics (e.g., aminoglycosides such as AK, apramycin, and streptomycin) were less so. Moreover, low-level intermediate resistance to newer generation cephalosporins was also

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observed. However, it is unclear whether antibiotic resistance patterns in environmental *Vibrio* isolates recovered from the Chesapeake Bay have changed over time.

To address this data gap, we conducted a longitudinal study to analyze trends in antibiotic resistance of *V. parahaemolyticus* and *V. vulnificus* isolates collected from Chesapeake Bay waters during two 3-year sampling periods that took place a decade apart (2009–2012 and 2019–2022). Of particular interest were the potential antimicrobial resistance patterns associated with changes in environmental parameters or the presence of virulence factors.

RESULTS

Water quality parameters

The average water temperature, salinity, pH, and dissolved oxygen (DO) concentrations were relatively uniform between longitudinal sampling periods (2009–2012 and 2019–2022) during the summer season (June, July, and August) in Tangier Sound (Table 1). In contrast, chlorophyll-*a* concentrations were notably higher between 2019 and 2022, but differences could not be directly compared due to the use of different analysis methods between sampling periods (Table 1).

During the 2009–2012 sampling period, DO, and chlorophyll-*a* concentrations were not significantly different between months. Whereas water temperature, salinity and pH were significantly different across each month ($P < 0.001$). The highest average water temperature, salinity and pH were recorded during the month of August, while the lowest were recorded during the month of June (Table 1). During the 2019–2022 sampling period, pH concentrations were not significantly different between months. Conversely, water temperature, salinity, DO, and chlorophyll-*a* were significantly different across each month ($P < 0.001$) (Table 1). The highest average water temperature and salinity were observed during the months of July and August, respectively, while the lowest were recorded during the month of June. Average DO and chlorophyll-*a* concentrations were highest during the month of June, and lowest during the month of August and July, respectively (Table 1).

Vibrio spp. virulence factors

Of the $n = 472$ *Vibrio* spp. isolates collected during the summer season in Tangier Sound for both sampling periods (2009–2012 and 2019–2022), 44% of *V. parahaemolyticus* ($n = 134$) and 55% of *V. vulnificus* ($n = 94$, 16 isolates could not be revived) isolates were selected for testing. All tested *V. parahaemolyticus* isolates were negative for the presence of *V. parahaemolyticus* associated virulence factors, thermostable direct hemolysin (*tdh*) and thermostable direct-related hemolysin (*trh*) (Table 2). The prevalence of *V. vulnificus* associated virulence factors varied between sampling periods, except for the pilus-type IV assembly gene (*pil*A) which was present in roughly the same percentage

TABLE 1 Water quality characteristics by sampling period (2009–2012 and 2019–2022) and month, including average temperature (T), salinity (S), pH, DO, and chlorophyll-*a* concentration (chla) with ±standard deviatio

Sampling period/month	T (°C)	S (ppt)	pH	DO (mg/L)	Chla (µg/L)
2009–2012					
June	25.5 ± 1.5	12.4 ± 2.1	7.4 ± 0.6	6.6 ± 1.0	12.8 ± 4.2
July	27.4 ± 1.0	13.8 ± 2.4	7.8 ± 0.4	7.0 ± 0.7	13.7 ± 4.5
August	28.0 ± 1.3	14.3 ± 1.9	8.0 ± 0.4	6.8 ± 0.4	12.3 ± 3.3
Overall	27.1 ± 1.6	13.6 ± 2.0	7.8 ± 0.5	6.8 ± 0.7	13.1 ± 3.6
2019–2022					
June	25.3 ± 0.0	11.4 ± 0.0	8.0 ± 0.0	7.6 ± 0.0	19.8 ± 0.0
July	29.1 ± 1.6	13.8 ± 3.1	7.9 ± 0.1	7.0 ± 0.3	13.7 ± 4.1
August	26.6 ± 2.5	15.4 ± 0.1	8.0 ± 0.1	6.6 ± 0.4	17.7 ± 2.5
Overall	27.1 ± 2.0	13.9 ± 2.3	8.0 ± 0.1	7.0 ± 0.5	16.8 ± 5.0

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TABLE 2 Number of selected *V. parahaemolyticus* and *V. vulnificus* isolates and prevalence of associated virulence factors (*V. parahaemolyticus*: *tdh*$^+$, *trh*$^+$; *V. vulnificus*: *vcg*C$^+$, *pil*A$^+$, *rtx*A$^+$) by sampling period (2009–2012 and 2019–2022) and month

Study/month	*V. parahaemolyticus*			*V. vulnificus*			
	N	*tdh*$^+$ (%)	*trh*$^+$ (%)	*N*	*vcgC*$^+$ (%)	*pilA*$^+$ (%)	*rtxA*$^+$ (%)
2009–2012							
June	24	0	0	10	—[a]	40	50
July	32	0	0	18	—	17	28
August	28	0	0	23	—	0	30
Overall	84	0	0	51	—	14	33
2019–2022							
June	12	0	0	5	0	20	0
July	17	0	0	13	15	8	0
August	21	0	0	9	22	22	11
Overall	50	0	0	27	15	15	4

[a]—, data not available.

of isolates during each sampling period (14% and 15% in 2009–2012 and 2019–2022, respectively) (Table 2). The prevalence of *pil*A was highest in isolates collected in June during the earlier sampling period (40%) and August during the later sampling period (22%). Virulence-correlated gene clinical variant (*vcgC*) was present in 15% of isolates recovered from 2019 to 2022, particularly during the month of August (22%), but data were not available for the isolates collected in 2009–2012 (Table 2). The prevalence of the composite toxin (*rtxA*) was much greater in isolates from the 2009–2012 sampling period (33%), especially in the month of June (50%), but it was present in only 4% of isolates collected during the later sampling period (2019–2022), and only during August (Table 2).

Antimicrobial resistance in *V. parahaemolyticus*

During the 2009–2012 sampling period, all *V. parahaemolyticus* isolates tested were fully susceptible to two antibiotics (CIP and SXT) out of the eight antibiotics tested (Fig. 1). During the 2019–2022 sampling period, all *V. parahaemolyticus* were fully susceptible to three antibiotics [imipenem (IPM), TE, and CIP]. Intermediate resistance was infrequently observed among the *V. parahaemolyticus* isolates tested, with the greatest intermediate resistance observed against AMP (2009–2012: 1%, 2019–2022: 4%) and amikacin (both sampling periods: 4%) (Fig. 1).

In terms of complete resistance, a high percentage of resistance was observed during both sampling periods for AMP (2009–2012: 94%, 2019–2022: 96%). Lower percentages of resistance were also seen against TE (2009–2012: 6%), CTX (2019–2022: 2%), CAZ (both sampling periods: 2%), AK (2019–2022: 2%), and CIP (2019–2022: 2%) (Fig. 1).

Antimicrobial resistance in *V. vulnificus*

During the 2009–2012 sampling period, all tested *V. vulnificus* isolates were susceptible to one antibiotic (CIP) out of the eight antibiotics tested (Fig. 2). During the 2019–2022 sampling period, all *V. vulnificus* were susceptible to seven antibiotics (AMP, CTX, IPM, AK, TE, CIP, and SXT) (Fig. 2).

Low levels of intermediate resistance were observed among the tested *V. vulnificus* isolates with regard to CAZ (2019–2022: 4%), IPM (2009–2012: 2%), AK (2009–2012: 2%), and TE (2009–2012: 4%) (Fig. 2). In terms of complete resistance, the 2019–2022 isolates did not express resistance against any of the antibiotics tested, while the 2009–2012 isolates expressed low levels of resistance to AMP (16%), CTX (6%), SXT (4%), CAZ (2%), and AK (2%) (Fig. 2).

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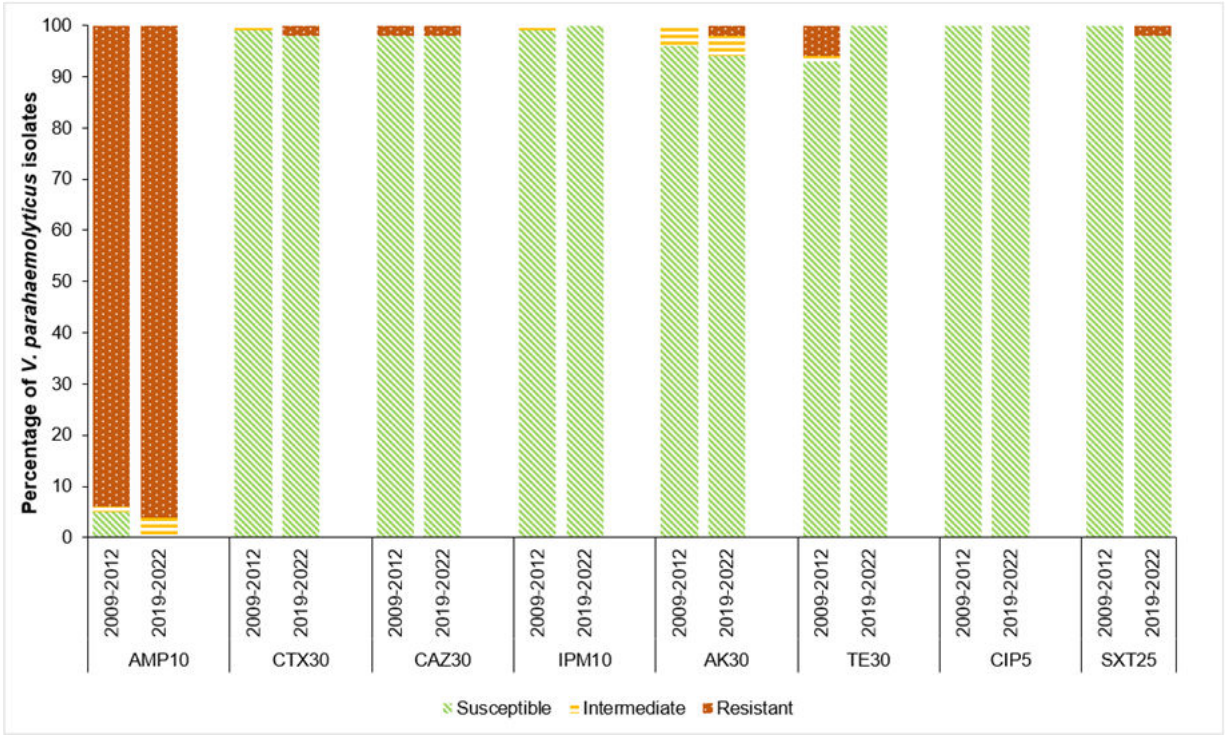

FIG 1 Antibiotic resistance patterns among *V. parahaemolyticus* isolates (*n* = 134). AK30, amikacin 30 µg; AMP10, ampicillin 10 µg; CAZ30, ceftazidime 30 µg; CIP5, ciprofloxacin 5 µg; CTX30, cefotaxime 30 µg; IPM10, imipenem 10 µg; SXT25, trimethoprim-sulfamethoxazole 25 µg; TE30, tetracycline 30 µg.

Multiple antibiotic resistance profiles for *Vibrio* spp

The overall percentage of multi-drug resistant (MDR) *V. parahaemolyticus* isolates, expressing resistance to two or more antibiotic classes, was similar between both sampling periods: 8% during 2009–2012 and 6% during 2019–2022 (Fig. 3). However, trends differed by month. August samples had a greater percentage of MDR *V. parahaemolyticus* isolates in the earlier sampling period (*n* = 14%), while in the later sampling period, the month of June, had the highest percentage of MDR isolates (*n* = 17%) (Fig. 3).

During the 2009–2012 sampling period, 8% of *V. vulnificus* isolates were MDR, with the greatest percentage of MDR isolates (20%) collected during the month of June, whereas the *V. vulnificus* isolates selected from 2019 to 2022 samples did not display multi-drug resistance (Fig. 3).

The multiple antibiotic resistance (MAR) index for isolated *V. parahaemolyticus* from both sampling periods (2009–2012 and 2019–2022, *n* = 134) ranged between 0 and 0.38, with an average of 0.13 and with 10 isolates (7%) exhibiting a MAR value greater than 0.2 (Table 3). Resistance to AMP was common for all 10 isolates and most frequently combined with resistance against TE (*n* = 5). Only one *V. parahaemolyticus* isolate was resistant to three antibiotics (AMP, CTX, and CAZ) with a MAR value of 0.38 (Table 3).

The MAR index for isolated *V. vulnificus* from both sampling periods (2009–2012 and 2019–2022, *n* = 78) also ranged between 0 and 0.38, with an average of 0.02 and with four *V. vulnificus* isolates (5%) exhibiting a MAR value >0.2 (Table 3). Resistance against AMP and CTX was observed in three out of four of these *V. vulnificus* isolates. A MAR value of 0.38 was observed in two *V. vulnificus* isolates with resistance against AMP, CTX, and SXT (Table 3).

MAR index analysis

The MAR index was not found to be statistically significantly different between the two sampling periods (2009–201 vs. 2019–2022) for *V. parahaemolyticus* isolates, of

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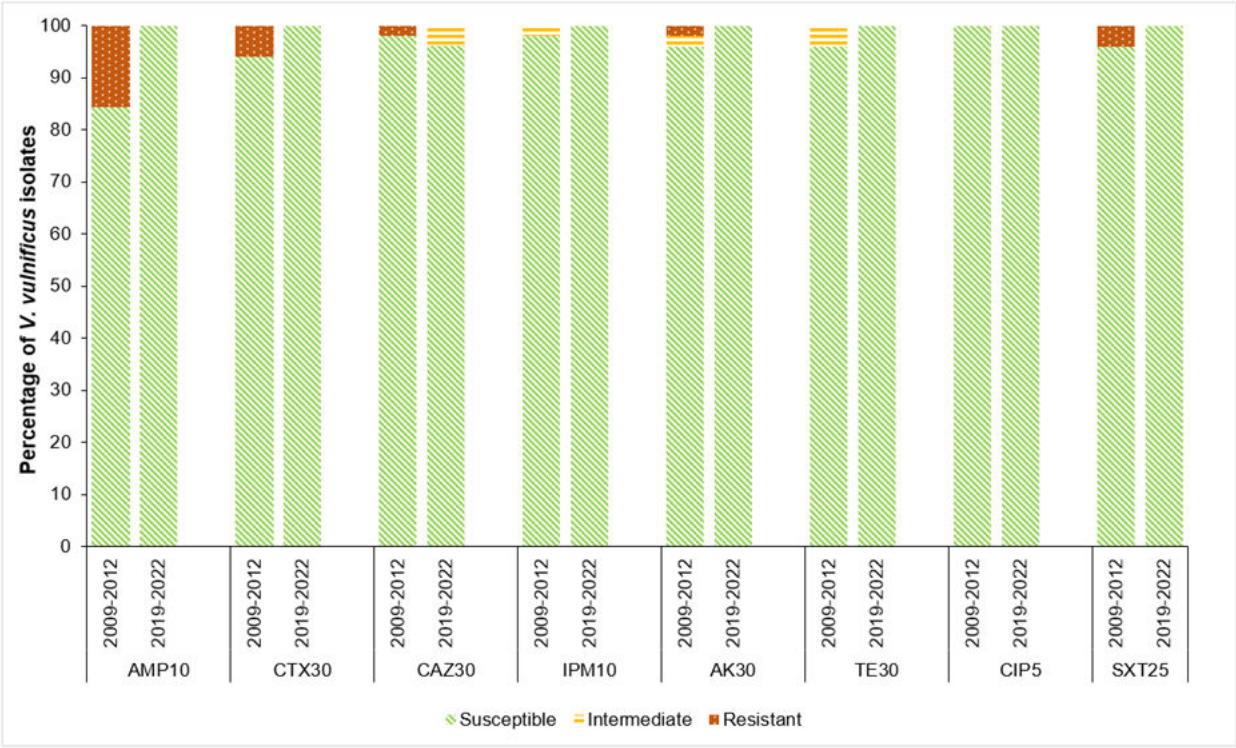

FIG 2 Antibiotic resistance patterns among *V. vulnificus* isolates (*n* = 78). AK30, amikacin 30 µg; AMP10, ampicillin 10 µg; CAZ30, ceftazidime 30 µg; CIP5, ciprofloxacin 5 µg; CTX30, cefotaxime 30 µg; IPM10, imipenem 10 µg; SXT25, trimethoprim-sulfamethoxazole 25 µg; TE30, tetracycline 30 µg.

those tested, but was statistically significantly different between sampling periods for *V. vulnificus* ($P = 0.004$); with the earlier sampling period characterized by a higher overall MAR index average. The sampling month did not significantly affect the MAR index among *V. parahaemolyticus* ($P = 0.707$) or *V. vulnificus* ($P = 0.541$). The sampling year did not significantly impact the MAR index among *V. parahaemolyticus* ($P = 0.982$) or *V. vulnificus* ($P = 0.065$).

Linear regression models developed for tested *V. parahaemolyticus* and *V. vulnificus* isolates, while controlling for year and month, did not yield statistically significant effects for any environmental water parameter (e.g., water temperature, salinity, DO, salinity, pH, chlorophyll-*a*), or combination of miscellaneous parameters on the MAR index ($P > 0.05$).

V. vulnificus virulence factors (*vcg*C, *pil*A, *rtx*A) and the MAR index, while controlling for year and month, were not significantly correlated nor did they display a significant linear relationship ($P > 0.05$).

DISCUSSION

Recent works (15, 37), reported a noted increase in potentially pathogenic environmental *Vibrio* spp. in Maryland coastal waters as well as an increase in vibriosis incidence rates in the state of Maryland since 2006, underscoring the need for further surveillance. This study represents the first long-term survey of antibiotic resistance among environmental *Vibrio* spp. isolates recovered from the Chesapeake Bay, Maryland. Consistent with an earlier study in Chesapeake Bay waters by Shaw et al. (22), a high percentage of AMP resistance was found in *V. parahaemolyticus* isolates during both sampling periods, 2009–2012 and 2019–2022, especially in the latter where all tested isolates expressed resistance (96%) or intermediate resistance (4%). As mentioned by Han et al. (21) and others (18, 21, 22), given the high levels of resistance found in *V. parahaemolyticus* against AMP, penicillin-based medicines are likely not effective as a single-use antibiotic to treat severe vibriosis and are no longer recommended by the CDC for this purpose

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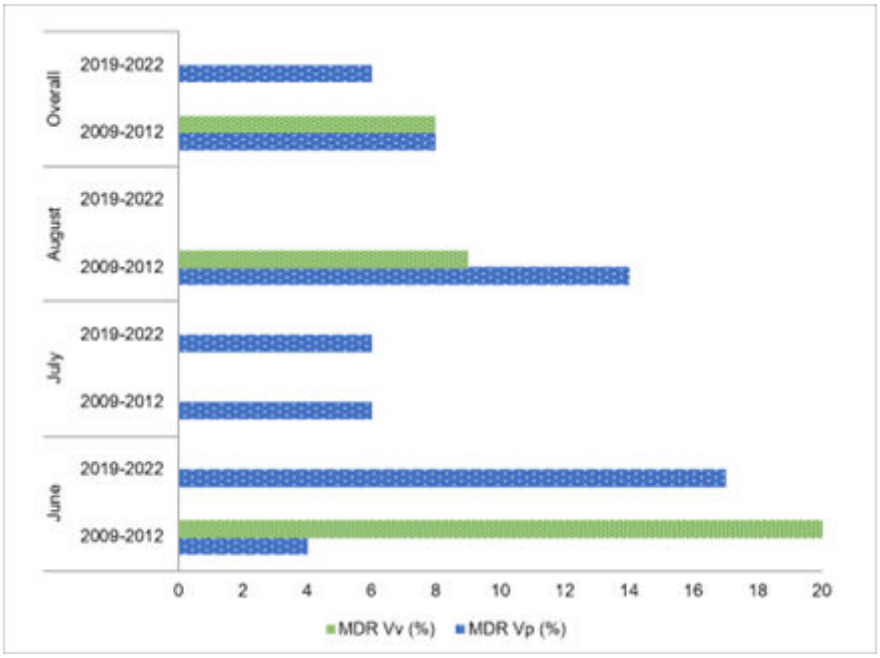

FIG 3 Percentage of MDR *V. parahaemolyticus* (Vp) and *V. vulnificus* (Vv) from each sampling period (2009–2012 and 2019–2022) and month.

(20). AMP resistance was also observed for *V. vulnificus* isolates in the current study, but only for samples collected during 2009–2012 (16%). This percentage was higher than previously reported for Chesapeake Bay waters in 2014, where only 1% of *V. vulnificus* isolates were resistant against AMP (22), and is closer to findings by Elmahdi et al. (26) where 26% of *V. vulnificus* recovered from oysters collected in Maryland in 2018 were AMP-resistant.

Of the CDC-recommended antibiotics used to treat severe vibriosis (TEs combined with third-generation cephalosporins, fluoroquinolones alone, and aminoglycosides combined with SXT), only fluoroquinolones demonstrated full efficacy against all *V.*

TABLE 3 MAR index[a] frequency among *V. parahaemolyticus* and *V. vulnificus* isolates and resistance profiles for both sampling periods (2009–2012 and 2019–2022) combined

Vibrio	Resistance profile[b]	MAR index	Frequency # (%)
V. parahaemolyticus			
N = 134	None	0.00	7 (5)
	AMP10	0.13	117 (87)
	AMP10, TE30	0.25	5 (4)
	AMP10, CAZ30	0.25	2 (1)
	AMP10, AK30	0.25	1 (1)
	AMP10, SXT25	0.25	1 (1)
	AMP10, CTX30, CAZ30	0.38	1 (1)
V. vulnificus			
N = 78	None	0.00	69 (88)
	AMP10	0.13	5 (6)
	AMP10, AK30	0.25	1 (1)
	CTX30, CAZ30	0.25	1 (1)
	AMP10, CTX30, SXT25	0.38	2 (3)

[a]The MAR index is calculated by dividing the number of antibiotics to which the isolate expressed resistance (*x*) by the total number of antibiotics to which the isolate was tested (*y*); a value >0.2 can be reflective of increased antibiotic contamination (36).
[b]Abbreviations used: AK30, amikacin 30µg; AMP10, ampicillin 10µg; CAZ30, ceftazidime 30µg; CTX30, cefotaxime; SXT25, trimethoprim-sulfamethoxazole 25µg; TE30, tetracycline 30µg.

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parahaemolyticus and *V. vulnificus* isolates tested (20). These findings are similar to previous studies on the East Coast of the U.S. that found that most or all environmental *Vibrio* spp. tested were susceptible to fluoroquinolones (22, 24, 32, 38), and it suggests, as recommended by Wong et al. (18), that fluoroquinolones should be considered by health care professionals as a first-line agent to treat severe and life-threatening vibriosis. Notwithstanding, increased levels of intermediate resistance and resistance to CIP (fluoroquinolone), ranging from 7% to 67%, have been documented for *V. parahaemolyticus*, *Vibrio alginolyticus,* and *Vibrio harveyi* isolated from seafood in Malaysia (30) and India (39) as well as from seawater samples in Brazil (31), indicating the ability for fluoroquinolone resistance to be acquired by *Vibrio* spp.

In terms of potential pathogenicity, all selected *V. parahaemolyticus* isolates tested negative for *tdh* and *trh* virulence genes. Although present in a large percentage of clinical strains, these genes are often found in <1% of all environmental strains (26, 40), and may not always reflect the bacteria's true ability to cause illness (41). Namely, Mahoney et al. (41) found that many environmental isolates lacking *tdh* and *trh* were still highly cytotoxic to human gastrointestinal cells and had the ability to horizontally acquire and regulate new virulence factors (41). In contrast, *V. vulnificus* isolates from both sampling periods tested positive for virulence factors believed to be important in causing disease, namely *vcg*C, *rtx*A, and *pil*A (42–45). Although the presence of these virulence factors may be significant, their absence does not preclude an isolate's ability to cause disease, as the mechanisms involved appear to be relatively complex and different virulence genes may act in combination with each other (9). In particular, while earlier studies found that the *vcg*C gene was more common in clinical strains capable of causing severe infection, a recent study found no difference in virulence between isolates carrying the *vcg*C gene, and those carrying the virulence correlated environmental gene (*vcg*E) (46–48). Nonetheless, the *vcgC* gene was present in 15% of the 2019–2022 isolates, which is similar to findings by Elmahdi et al. (26) and Warner and Oliver (49), who reported prevalence rates for the *vcg*C gene of 20.9% and 15.6%, respectively, among *V. vulnificus* recovered from oyster samples. *Pil*A was also found in approximately 15% of all environmental strains and at a similar rate during both sampling periods, while *rtx*A was more prevalent in the 2009–2012 isolates tested, 33% compared to only 4% for the 2019–2022 isolates. While a direct association between virulence factors and antimicrobial resistance has not been established, previous studies suggest that vibrios, including non-pathogenic strains, are able to simultaneously acquire virulence factors and antimicrobial resistance genes from other bacteria and their surrounding environment (50–52). In this study, we did not observe a significant correlation between the presence of virulence factors and the presence of MAR. However, our findings suggest that *V. vulnificus* isolates with the potential to cause disease may be prevalent in this important recreational and commercial watershed.

Comparing 2009–2012 to 2019–2022, the percentage of MDR *V. parahaemolyticus* isolates found was not significantly different between sampling periods, 8% and 6%, respectively. Interestingly, the number of MDR strains observed increased throughout the summer season during the earlier sampling period but decreased in the latter, which may warrant further study into possible seasonal trends and potentially temperature-regulated antibiotic resistance. Multi-drug resistance in *V. vulnificus* was also found in <10% of isolates but was observed only for samples from 2009 to 2012 and not for the later sampling period. Nonetheless, the absence of MDR *V. vulnificus* isolates between 2019 and 2022 may not be indicative of a decreasing trend in the lower Chesapeake Bay, but rather a result of the limited number of isolates tested (n = 16 could not be revived). Overall, these results were similar to those observed in Maryland Coastal Bays by Shaw et al. (22) and Elmahdi et al. (26) but lower than findings from Da Silva et al. (32) and Baker-Austin et al. (24), where approximately 40% of *V. parahaemolyticus* and *V. vulnificus* isolates were characterized by multi-drug resistance. Notwithstanding, the higher levels of multi-drug resistance observed in these previous studies may be related to the greater

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number of antibiotics tested (around 20), the analysis of multiple environmental sample types, and the selection of industrially contaminated sites.

Importantly, the percentage of isolates displaying a MAR index >0.2 was relatively low for both *V. parahaemolyticus* and *V. vulnificus* from both sampling periods combined, namely 7% and 5%, respectively. Furthermore, only one *V. parahaemolyticus* isolate and two *V. vulnificus* isolates were resistant against more than two antibiotics, which included AMP, 3rd generation cephalosporins, and SXT. As mentioned previously, a MAR index >0.2 is indicative of an area with increased sources of antibiotic contamination in the environment, and a greater likelihood the spread of antibiotic resistance genes among bacterial pathogens (31, 36). Although Tangier Sound is adjacent to a land area with a history of heavy agricultural use, ranking first in the State of Maryland for broiler poultry production (53), our findings did not demonstrate the same high prevalence of antimicrobial resistance as in other comparable sites (22, 24, 32). However, it should be noted that Tangier Sound, located in the lower Chesapeake Bay, may benefit from increased tidal flow compared to other sites in the upper and mid-Bay or more inland waterways (54) and may not be representative of conditions across the Bay or throughout the seasons.

Limitations of our study included selection of *Vibrio* spp. isolates collected only during peak vibrio abundance season and from one station in the lower Bay, which restricted our ability to analyze antimicrobial resistance trends throughout the year and across the estuary. Moreover, our analysis was based on isolates recovered from water samples processed using culture-dependent methods with selective media, which has been shown to be less sensitive in detecting virulence factors by PCR, compared to real-time PCR with enrichment (55). In addition, chlorophyll-*a* levels in each sampling period (2009–2012 and 2019–2022) were measured using different instruments and methodology, which could have influenced the differences observed. Of the *V. vulnificus* isolates selected from the 2019 to 2022 sampling period, 16 could not be revived and our results may underrepresent changes to antimicrobial resistance during this sampling period. Finally, another limitation of our study is the lack of continuous sampling between longitudinal studies, which led to a 10-year gap in antibiotic resistance data.

Conclusions

The presence of virulence genes and the prevalence of antimicrobial resistance in environmental *V. vulnificus* and *V. parahaemolyticus* isolates have a direct impact on the prevention and management of vibriosis. Although the percentage of multi-drug resistance observed in our study was relatively low, the isolates that were tested showed varying levels of resistance and intermediate resistance to antibiotics typically used to treat severe vibriosis, including third-generation cephalosporins, TEs, SXT, and aminoglycosides. Exceptionally, all isolates were susceptible to CIP, a fluoroquinolone. *V. vulnificus* isolates also carried multiple virulence factors found in disease-causing pathogenic strains. Thus, prompt diagnosis and treatment by health care professionals with an effective antibiotic, for example, fluoroquinolones, are imperative for severe vibrio infections incurred from exposure to lower Chesapeake Bay waters and possibly across the Bay. Overall, our results show that antibiotic resistance patterns among *V. parahaemolyticus* and *V. vulnificus* recovered from the lower Chesapeake Bay have remained relatively stable since 2009.

MATERIALS AND METHODS

Site description and source selection

Water samples were collected from Tangier Sound in the Chesapeake Bay, Maryland, USA, during two separate 3-year sampling events, namely 2009–2012 and 2019–2022, in previous studies (37, 56). For spatial-temporal comparison purposes, only isolates from water samples collected during the summer months (June, July, August) were selected

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for this study. Tangier Sound (38°10.97′N, 75°57.90″W) is a mesohaline region of the lower Chesapeake Bay, just west of Maryland's southernmost bay county of the Eastern Shore (Somerset County) and close to the Virginia border (Fig. 4).

The land area adjacent to Tangier Sound is characterized by heavy agricultural use and ranks first in the State of Maryland for broiler poultry production (53). Tangier Sound is also a popular location for recreational and commercial fishing, including crabbing and oyster harvesting (57).

Sample collection and processing

Methods used for sample collection and processing have been previously described by Brumfield et al. (37), Chen et al. (56), and Johnson et al. (58, 59); a summary of methods related to this study are provided here. Physical and chemical measurements were collected during each sampling event using a handheld water probe (Eureka, Austin, TX, USA), including water temperature, pH, DO, salinity, and chlorophyll-*a*. Total chlorophyll-*a* concentration for the 2009–2012 sampling period was measured in methanol extracts on a Cary model 50 UV-visible-light spectrophotometer, whereas it was measured in acetone extracts on a Shimadzu UV 2401PC spectrophotometer for the 2019–2022 sampling period. Water samples (12 L) were collected just below the surface, transported back to the laboratory on ice, and kept refrigerated overnight until processing the following morning. Collected water was shaken, and three volumes of water (1000, 100, and 10 mL) were inoculated into differing volumes of 10× alkaline peptone water (APW, pH 8.5) to achieve an inoculated concentration of 1X APW. The 1× APW solutions were then incubated at 33°C for 16–18 h. with shaking at 30 rpm. Following incubation, a loopful of pellicle was removed from each enriched sample and streaked individually into selective media, including CHROMagar *Vibrio* (CHROMagar, Springfield, NJ, USA), thiosulfate citrate bile salts sucrose agar (TCBS, Oxoid, Ontario, Canada), and *V. vulnificus*

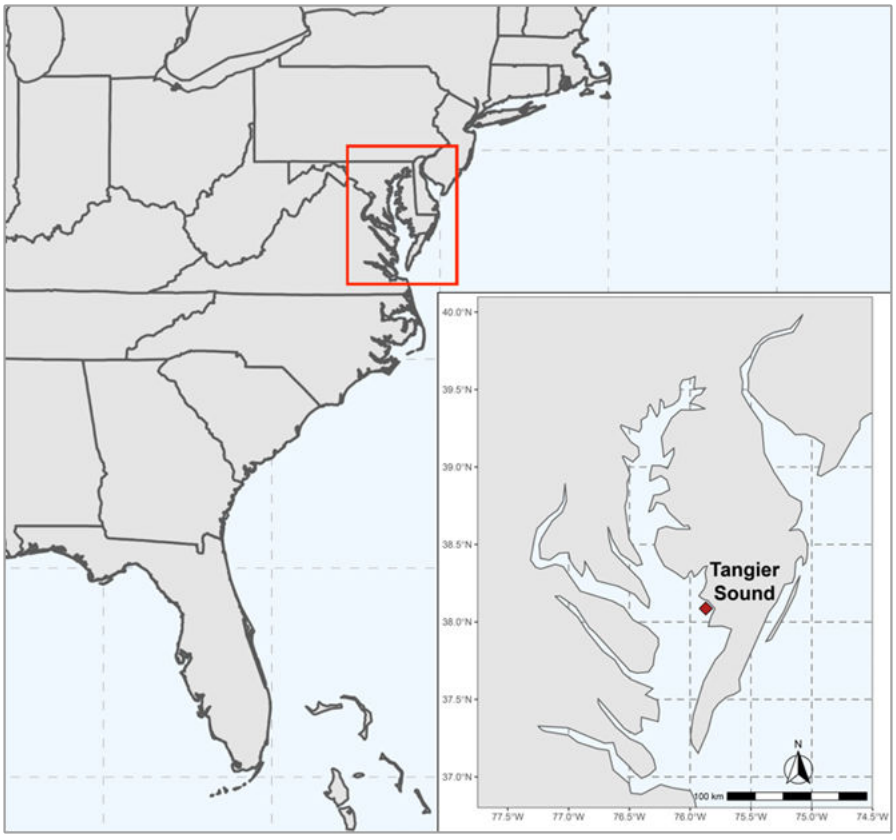

FIG 4 Map of the Chesapeake Bay showing sampling site in Tangier Sound.

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agar [VVA; 2% peptone, 3% NaCl, 1% cellobiose, 0.06% bromothymol blue (pH 8.2)]. The plates were incubated at 37°C for 16–18 h and presumptive colonies of *V. parahaemolyticus* and *V. vulnificus* were picked and streaked onto Difco Luria-Bertani (LB) agar (Becton, Dickinson and Co., Sparks, MD, USA) to obtain pure cultures. Pure culture isolates were grown overnight in undiluted LB broth and added to equal volume 50% glycerol and stored at −80°C.

Vibrio species confirmation

DNA was extracted from presumptive isolates of *V. parahaemolyticus* and *V. vulnificus* following methods described in Chen et al. (56) and confirmed using PCR targeting the *tox*R gene adapted from Bauer and Rørvik (60) to differentiate between the two vibrios. Additionally, samples testing positive for either species were further tested for species-specific genes and virulence markers [*V. parahaemolyticus*: thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*), thermostable direct-related hemolysin (*trh*); *V. vulnificus*: hemolysin cytolysin (*vvha*), virulence-correlated gene environmental variant (*vcg*E), virulence-correlated gene clinical variant (*vcg*C), RTX toxin (*rtx*A), type IV pili (*pil*A)]. *Vcg*C$^+$ data were not available for *V. vulnificus* isolates selected from the 2009–2012 longitudinal study. PCR assays were performed using Promega GoTaq Green Master Mix 2× (Promega, Madison, WI, USA); each reaction well contained a total of 25 µL, including 20 µL of master mix solution (12.5 µL of GoTaq, 1 µL of each primer, and nuclease-free water to reaction volume) and 5 µL DNA template. The primer sequences, amplicon sizes, and conditions used for each PCR assay can be found in Table 4.

PCR products were stored at 4°C until gel electrophoresis visualization. Positive controls included *V. parahaemolyticus* ATCC 17803 (*tox*R$^+$), NIHCB0757 (*tlh*$^+$/*tdh*$^+$), and AQ 4037 (*tlh*$^+$/*trh*$^+$); and *V. vulnificus* ATCC 27562 (*tox*R$^+$/*vcg*E$^+$/*vvh*A$^+$), and ATCC 29307 (*vcg*C$^+$/*pil*A$^+$/*rtx*A$^+$). Nuclease-free water was used as a negative control in each reaction. PCR products were stained using BioLink Smart Glo Pre stain and visualized using a 1.5% agarose gel at 110 V for 60–90 min and viewed under a UV transilluminator using a Gel Documentation System (GelDoc-IT, UVP, LLC, CA, USA).

Antimicrobial susceptibility testing

A total of *n* = 134 *V. parahaemolyticus tox*R-confirmed isolates (*n* = 84 from 2009 to 2012; *n* = 50 from 2019 to 2022) and *n* = 94 *V. vulnificus tox*R-confirmed isolates (*n* = 51 from 2009 to 2012; *n* = 43 from 2019 to 2022) were subjected to antimicrobial susceptibility testing. These isolates were randomly selected, using the RAND function in Excel, from samples recovered from Tangier Sound during the summer season (June, July, and August) during both sampling periods. All tested isolates were also positive for their respective species identifying markers, namely the *tlh* marker (*V. parahaemolyticus*) and the *vvhA* marker (*V. vulnificus*). Isolates kept at −80°C were streaked onto LB agar (Miller, USA) plates and incubated at 37°C between 16 and 18 overnight. The concentration of *Vibrio* spp. suspensions was adjusted to a 0.5 McFarland standard using a nephelometer. Antibiotic susceptibility testing was carried out using the Kirby-Bauer disk diffusion method on Muller–Hinton (MH) agar (BD, USA), according to Clinical and Laboratory Standards Institute guidelines for *Vibrio* spp (71). and *Enterobacteriaceae* (72). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains; pure water was used as a negative control.

All isolates were tested for susceptibility to eight antibiotics from seven different antibiotic classes frequently used to treat severe *Vibrio* spp. infections (18). This included AMP (10 µg), CTX (30 µg), CAZ (30 µg), IPM (10 µg), AK (30 µg), TE (30 µg), CIP (5 µg), and SXT (23.75 and 1.25 µg, respectively). The MAR index was calculated by dividing the number of antibiotics to which the isolate expressed resistance (*x*) by the total number of antibiotics to which the isolate was tested (*y*) (36). A MAR index >0.2 suggests an area with increased sources of antibiotic contamination in the environment, and a greater likelihood for the spread of antibiotic resistance genes among bacterial pathogens (31, 36). Isolates were further classified as MDR when they expressed resistance to two or

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TABLE 4 List of primer sequences, amplicon sizes, and PCR conditions used for the detection of *V. parahaemolyticus* and *V. vulnificus* species-specific genes and virulence genes

Primer	Primer sequence (5′–3′)	Amplicon (bp)	PCR conditions	Source
utox-F	GASTTTGTTTGGCGYGARCAAGGTT			(60)
vptox-R	GGTTCAACGATTGCGTCAGAAG	297	95°C for 4 min; 34×: 95°C for 30 s, 55°C for 30 s, 72°C for 60 s; 72°C 5 min	
vvtox-R	AACGGAACTTAGACTCCGAC	435	95°C for 4 min; 30×: 95°C for 30 s, 60°C for 30 s, 72°C for 60 s; 72°C 7 min	
tlh-F	AAAGCGGATTATGCAGAAGCACTG	173	94°C for 3 min; 30×: 94°C for 60 s, 58°C for 60 s, 72°C for 60 s; 72°C 5 min	(61, 62)
tlh-R	TGTGCCTTGATGAACTCGTTC			
tdh-F	GTAAAGGTCTCTGACTTTTGGAC	270		
tdh-R	TGGAATATGAACCTTCATCTTCACC			
trh-F	TTGGCTTCGATATTTTCAGTATCT	500		
trh-R	CATAACAAACATATGCCCATTTCCG			
vvh-F	AGCGGTGATTTCAACG	411	94°C for 3 min; 34×: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; 72°C 5 min	
vvh-R	GGCCGTCTTTGTTCACT			
vcgC-F	AGCTGCCGATAGCGATCT	97	94°C for 3 min; 30×: 94°C for 40 s, 57°C for 40 s, 72°C for 40 s; 72°C 5 min	(49, 63–66)
vcgC-R	TGAGCTAACGCGAGTAGTGAG			
vcgE-F	CTCAGAAAGGCTCAATTGAC	199		
vcgE-R	GATTAACGCTGTAAGGCCG			
pilA-F	TGGCTGCTGTTGCTATTC	217	94°C for 3 min; 30×: 94°C for 60 s, 60°C for 60 s, 72°C for 60 s; 72°C 5 min	(67, 68)
pilA-R	GGTCCACCACTAGTACCAAC			
rtxA-F	CGGGATCCTATGGCGTGAACGGCGAAG	1,440	94°C for 3 min; 30×: 94°C for 30 s, 68°C for 30 s, 72°C for 60 s; 72°C 5 min	(67, 69, 70)
rtxA-R	CGGGATCCAGCAGCCACAAGCGATTC			

more antibiotic classes. Isolates that could not be revived during antimicrobial susceptibility testing were omitted from the data analysis (n = 16 *V. vulnificus* from the 2019–2022 study period).

Data analysis

The Wilcoxon rank sum test and the Kruskal–Wallis test were used to assess whether the environmental water parameters and the MAR index of *V. parahaemolyticus* and *V. vulnificus* isolates measured in each longitudinal sampling period (2009–2012 vs. 2019–2022) and month differed significantly from each other. Linear regression models were also developed to characterize the effect of sampling month, year, and environmental water parameters (i.e., water temperature, salinity, pH, DO, and chlorophyll-*a*) on the MAR index in both studies combined. Data were aggregated by month for both *V. parahaemolyticus* and *V. vulnificus*, and models were assessed by adding each environmental water parameter at a time and evaluating the estimates of regression coefficients, and *P*-values. Correlation and regression analyses were performed to examine the relationship between *V. vulnificus* virulence markers (*Vcg*C, *pil*A, *rtx*A) and the MAR index. Statistical analyses were performed using SAS 9.4 (Cary, NC, USA).

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DATA AVAILABILITY

Data used to create figures and tables are available upon request.

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