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## **Monitoring and preventing foodborne outbreaks: are we missing wastewater as a key data source?**

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## Abstract

In 2022, the number of foodborne outbreaks in Europe increased by 43.9%, highlighting the need to improve surveillance systems and design outbreak predictive tools. This review aims to assess the scientific literature describing wastewater surveillance to monitor foodborne pathogens in association with clinical data. In the selected studies, the relationship between peaks of pathogen concentration in wastewater and reported clinical cases is described. Moreover, details on analytical methods to detect and quantify pathogens as well as wastewater sampling procedures are discussed. Few papers show a statistically significant correlation between high concentrations of foodborne pathogens in wastewater and the occurrence of clinical cases. However, monitoring pathogen concentration in wastewater looks like a promising and cost-effective strategy to improve foodborne outbreak surveillance. Such a strategy can be articulated in three steps, where the first one is testing wastewater with an untargeted method, like shotgun metagenomic, to detect microorganisms belonging to different domains. The second consists of testing wastewater with a targeted method, such as quantitative polymerase chain reaction, to quantify those specific pathogens that in the metagenomic dataset display an increasing trend or exceed baseline concentration thresholds. The third involves the integrated wastewater and clinical data analysis and modeling to find meaningful epidemiological correlations and make predictions.

## Introduction

In 2022, across the 27 member states of the European Union and in the United Kingdom, a total of 5763 foodborne outbreaks, involving 48,605 human cases, have been reported, resulting in 2783 hospitalizations and 64 deaths (EFSA and ECDC, 2023). Most individuals were exposed to contaminated food in domestic and public settings, including pubs, cafés, restaurants, hotels, bars, and catering services (Sarno *et al.*, 2021). The causative agents of 1014, 332, and 255 of the outbreaks that occurred in 2022 included *Salmonella*, norovirus (and other caliciviruses), and *Campylobacter*, respectively. Foodborne outbreaks are identified through epidemiological investigations, starting with passive case finding. Microbiological investigations of food and environmental samples for detecting the outbreak source are performed with molecular subtyping techniques (Swaan *et al.*, 2010; Nicolay *et al.*, 2011). Nevertheless, incomplete information, difficulties in assessing the evidence linking a specific food to clinical cases, privacy restrictions, possible unavailability of individuals to provide consent for the use of their samples, or variability in individual responses to foodborne pathogens lead to outbreak underreporting (Haagsma *et al.*, 2013; Gibbons *et al.*, 2014).

Passive epidemiological surveillance systems, on the one hand, show limitations in dealing with the appearance of new pathogens and the re-emergence of infections that were previously under control (Prado *et al.*, 2022), whereas, on the other hand, they may under-represent the spread of an outbreak due to errors in diagnosing, classification, or notifications of cases (WHO, 2021). Innovative surveillance and early warning techniques could help to ascertain the real burden of foodborne outbreaks and, to a certain extent, prevent them or provide data to contrast them through appropriate health policies. In this respect, wastewater-based epidemiology (WBE), which is an epidemiological approach involving the analysis of human wastewater, could be a valuable monitoring and predictive tool as it is capable of delivering objective, cost-effective, and comprehensive nearly real-time assessments of a community's health status (Robins *et al.*, 2022). Wastewater constitutes a reservoir of human fecal and environmental microorganisms, also comprising foodborne pathogens. Every day, wastewater treatment plants (WWTP) receive tons of wastewater from various sources, including private houses, hospitals, livestock farms, and food industries (García-Aljaro *et al.*, 2019). According to the WBE approach, which relies on the premise that a correlation exists between the concentration of a substance excreted in feces or urine by a population over a specified span of time and the concentration found in wastewater entering a WWTP during the same period (Prado *et al.*, 2022), wastewater analysis can provide data regarding a whole population in the WWTP catchment area.

In the last few years, wastewater analysis has been largely applied to trace the spreading of SARS-CoV-2 (Chau *et al.*, 2022). Several studies have shown that the curves of weekly reported trends in

SARS-CoV-2 viral load obtained through wastewater analysis overlapped with those regarding the number of active infection cases recorded at hospitals (Nattino *et al.*, 2022; Prado *et al.*, 2022; Maida *et al.*, 2023). In the context of SARS-CoV-2, WBE facilitated the implementation of early warning health policies. In a review, it is reported that the assessment of the circulation of the virus occurred up to 63 days before the onset of clinical cases in a community (Prado *et al.*, 2022).

Based on the achievements of WBE during the SARS-CoV-2 pandemic, the approach is now beginning to be applied also to predict and monitor community-wide exposure to foodborne pathogens (Hellmér *et al.*, 2014). This review aims to assess the scientific literature describing the application of wastewater surveillance to monitor foodborne pathogens in association with clinical data. Moreover, the analytical methods to detect and quantify the selected pathogens, as well as water sampling procedures, are also screened.

## Materials and Methods

A literature search was performed on the 19<sup>th</sup> of October 2023, using four databases (*i.e.*, Scopus, PubMed, Web of Science, CAB Abstracts). The search strategy was intended to identify i) studies using wastewater testing to detect foodborne pathogens, with particular focus on *Salmonella* spp., *Campylobacter* spp., *Escherichia coli*, *Listeria* spp., *Yersinia* spp., and Norovirus; ii) studies focusing on wastewater tested before entering the treatment plants; iii) studies correlating wastewater data to clinical data.

The following strings were used in the search: i) (sewage OR wastewater) AND (*Salmonella* OR salmonellosis OR *Campylobacter* OR campylobacteriosis OR *Escherichia coli* OR STEC OR escherichiosis OR *Listeria* OR listeriosis OR *Yersinia* OR yersiniosis OR Norovirus OR Norovirus infection) AND (detection) AND (human wastewater OR human sewage); ii) (sewage OR wastewater OR wastewaters) AND (surveillance OR monitoring OR epidemiology OR analysis OR identification OR detection OR wastewater-based epidemiology OR wastewater epidemiology OR sewage epidemiology OR wastewater surveillance OR public health) AND (foodborne pathogens OR foodborne microorganisms OR foodborne bacteria OR foodborne viruses OR pathogens).

The inclusion criteria were the English language and publication in the time span 2012-2023. Both research papers and reviews were retrieved, for a total amount of 3976 articles. After manually reviewing titles and abstracts, papers discussing only wastewater purification techniques, those focusing exclusively on SARS-CoV-2, and those addressing metatranscriptomics, applied in contexts other than food safety, were excluded.

## Results and Discussion

A total of 3976 papers were retrieved by adding the results of each search string in Scopus (55), PubMed (1091), Web of Science (2819), and CAB Abstracts (11). Duplicated papers were manually eliminated. A screening of the remaining papers was performed based on the reference to foodborne pathogens, both viruses and bacteria, and wastewater in the title, in the abstract, or in the full text, with the result of reducing the relevant body of literature to 42 papers (*Supplementary Table 1*). Among these papers, 24 referred to viruses, mostly norovirus, followed by hepatitis A virus (HAV), adenovirus, astrovirus, and polyomavirus. Moreover, 17 papers focused on bacteria, mostly *Salmonella*, followed by *Campylobacter*, *E. coli*, and *Shigella*. Out of the 42 selected papers, only seven correlated data on the concentration of foodborne pathogens in wastewater and clinical data and are described below.

### ***Foodborne pathogens detected in wastewater***

#### ***Viruses***

A total of four of the selected papers report a correlation between the viruses detected/quantified in the wastewater and clinical cases. The first paper refers to a study performed in Gothenburg in which the authors report a high concentration of norovirus in pooled samples of wastewater two to three weeks before clinical cases and high concentrations of HAV five to 13 weeks before clinical cases

(Hellmér *et al.*, 2014). During the epidemiological investigation, contaminated frozen red berries were identified as a possible cause of the outbreak. The authors compared the genetic composition of strains detected in wastewater and those isolated from sporadic clinical cases and from outbreaks of human samples, concluding that the HAV outbreak began in Denmark in the autumn of 2012 and spread throughout Scandinavia during the winter of 2012 and spring of 2013. In addition, by quantifying HAV RNA viral load in wastewater and comparing its concentration and genetic composition to clinical samples, researchers hypothesize that there were more infected people excreting viruses, than the population diagnosed by the health system, which indicated the presence of asymptomatic individuals, shedding the virus or simply not detected by the health system for other reasons (Hellmér *et al.*, 2014), demonstrating the monitoring effectiveness of the wastewater approach.

The second paper reports a study conducted over 3 years (2013-2016) in Japan by Kazama *et al.* (2017), who demonstrated the usefulness of monitoring noroviruses in wastewater for the sensitive detection of epidemic variants in human populations. The analysis of wastewater samples was employed to study norovirus concentrations and circulating genotypes in the population affected by gastroenteritis. Over the study period, a total of 296 rectal swab samples were obtained from patients suffering from gastroenteritis. Out of the samples analyzed, 102 tested positive for norovirus. Among these, 14 cases were caused by GI and 92 cases by GII. The GII concentration in wastewater showed a significant correlation ( $R=0.51$ ) with gastroenteritis cases without any temporal delay, indicating that the fluctuations in the concentration of the GII virus in wastewater coincided temporally with the fluctuations in gastroenteritis cases. Therefore, changes in norovirus GII concentration in sewage occurred synchronously with the number of infected patients. A total of 15 norovirus genotypes were identified in wastewater, 13 of which were also identified in human fecal samples.

In the third paper, the effectiveness of sewage surveillance was demonstrated by La Rosa *et al.* (2014) in a study on the spread of HAV across seven Italian regions by correlating direct RNA measurement from human wastewater and available clinical data. The study spanned 15 months, from July 2012 to September 2013, including the HAV outbreak that occurred in 2013, attributed to the consumption of frozen mixed berries. Environmental surveillance played a key role in the epidemiological investigation, which included the analysis of sewage samples from 19 WWTPs located in the seven regions most affected by the epidemic. The results showed that out of 157 wastewater samples, 24.2% were positive for HAV, with 16 positive samples collected in 2012 and 22 in 2013. Various HAV strains, including the IA variant responsible for the outbreak, were detected in sewage, and it was found some correlation between these isolates and isolates from clinical cases during the same period. Additionally, a prevalent IB strain with countrywide distribution was identified in sewage samples (9.4%), yet absent in clinical samples, implying, according to the authors, a silent circulation of the virus and potential surveillance deficiencies. The comparison of HAV-positive sewage samples with clinical notification data from corresponding WWTP catchment areas provided compelling evidence of HAV circulation in the population, without concurrent clinical notifications (La Rosa *et al.*, 2014). However, research findings did not offer alert signals for the prediction of outbreaks.

In their article, Kuhn *et al.* (2023) carried out a study for the long-term monitoring of norovirus, *Salmonella*, and *Campylobacter* in the human population in the Oklahoma state in the US. In this section, the results related to norovirus are reported, while results on *Salmonella* and *Campylobacter* are detailed below. The investigation involved the quantification of norovirus in the samples from 32 WWTP serving 1.8 million people from 2021 to 2022. The average weekly norovirus concentration was quantified in 390 million viral particles per liter, with a peak of 4.5 billion in February 2022. The highest concentration of norovirus was determined in autumn and winter, but no statistically significant correlation between season and the presence of the virus was identified in the dataset. The researchers determined an outbreak threshold within each season, corresponding to the 95th percentile of pathogen concentration in sewage. Beyond this threshold, a potential outbreak of infection was considered to possibly occur. Although the wastewater concentration of norovirus exceeded the 95th percentile seasonal threshold for seven weeks, no correspondence with officially reported clinical

cases with norovirus as a causative agent was found. However, since norovirus is not considered a state-reportable condition in Oklahoma, routine surveillance data may not capture all cases. The timing of confirmed outbreaks of norovirus was instead collected through information provided by the Centers for Disease Control and Prevention, and notably, during 5 out of the 7 weeks of norovirus outbreak alerts in wastewater, confirmed gastrointestinal outbreaks of unknown causes occurred in Oklahoma. Since 71% of the outbreaks with an unknown causative agent temporally coincided with peaks of norovirus in wastewater, the authors suggest a potential correlation between the virus and these outbreaks.

### *Salmonella*

*Salmonella* was investigated in four papers correlating wastewater and clinical data and in one article where clinical results were missing. The first paper refers to a study running for 54 weeks (April 27, 2010, to May 9, 2011) and monitoring the concentration of *Salmonella* in municipal wastewater samples from Honolulu, Hawaii. The results analyzed using the Pearson correlation coefficient showed that *Salmonella* concentration in wastewater samples was positively correlated with the number of clinical salmonellosis cases ( $p=0.006$ ) (Yan *et al.*, 2018). The concentration of *Salmonella* varied considerably during the sampling period, ranging between 105.5 and 106.8 CFU/100 mL, and these concentrations are in agreement with previous reports from different countries, including France, Finland, and Mexico. A total of 378 *Salmonella* isolates obtained from the wastewater were serotyped and characterized by pulsed-field gel electrophoresis (PFGE) along with 338 clinical isolates. Overall, 21 serotypes and 24 pulsotypes were shared between wastewater and clinical isolates. 9 of these shared pulsotypes, all associated with *S. Paratyphi B* isolates, showed concurrent detection, meaning they were identified within one week. While the presence of *S. Paratyphi B* in wastewater is not surprising, the authors were also able to detect the concurrent presence of a rare serotype, *S. Uganda*, in both clinical and wastewater isolates, demonstrating the feasibility of wastewater monitoring as an additional surveillance strategy for enteric diseases. However, the authors acknowledge the presence of data outliers affecting the use of this strategy as an early warning system.

In a later study, Diemert and Yan (2019) analyzed 63 isolates of *S. Derby* collected in wastewater in the study by Yan *et al.* (2018) to understand why this serotype was associated with as much as 21% of the isolates from wastewater and only 2% of the clinical isolates. The results showed that the serovar Derby has a low virulence potential in comparison to other serotypes while it is able to persist in wastewater better than other serotypes.

In their article, Yanagimoto *et al.* (2020) investigated, in the same time frame and locations, 689 *Salmonella* isolates from wastewater, classified in 38 serotypes, and 77 human isolates, classified in 26 serotypes. A total of 12 serotypes, including *S. Agona*, *S. Bareilly*, *S. Bovismorbificans*, *S. Brandenburg*, *S. Colindale*, *S. Infantis*, *S. Litchfield*, *S. Mbandaka*, *S. Newport*, *S. Saintpaul*, *S. Schwarzengrund* and *S. Stanley* were shared between wastewater and human isolates collected within one month. Moreover, checking epidemiological information and PFGE profile, six wastewater isolates belonging to five serotypes (*i.e.*, Anatum, Bovismorbificans, Colindale, Schwarzengrund, and Newport) were identified as closely related to human isolates (Yanagimoto *et al.*, 2020).

The fourth paper reports the study conducted by Kuhn *et al.* (2023) in Oklahoma described above. As for Norovirus, also for *Salmonella*, the authors compared bacterial concentration exceeding the 95th percentile seasonal threshold with notifications of confirmed *Salmonella* outbreaks. In the study period, 1157 *Salmonella* cases were reported across 47 weeks. Notably, in 11 out of these 47 weeks, the *Salmonella* concentration exceeded the 95th percentile seasonal threshold in sewage and in the same period, the exceeding of the seasonal threshold in sewage coincided with confirmed *Salmonella* cases reported one week later. Therefore, the authors demonstrate that the concentration of *Salmonella* in wastewater can serve as an early warning indicator of potential outbreaks with an advantage of at least one week (Kuhn *et al.*, 2023). Moreover, the study illustrates that the *Salmonella*

concentration threshold in wastewater provides a robust measure for assessing the risk to which a population is exposed and helps to guide actions for the management and prevention of salmonellosis.

### *Campylobacter*

During the literature search, two studies regarding *Campylobacter* in wastewater and reporting also clinical data were retrieved (Kuhn *et al.*, 2023; Zhang *et al.*, 2023). Zhang *et al.* (2023) developed an innovative triplex real-time quantitative polymerase chain reaction (qPCR) assay which demonstrated high sensitivity and specificity for both *C. jejuni* and *C. coli* quantified in the wastewater at a minimum concentration of 2 log<sub>10</sub> cells/mL. In detail, the authors tested 52 raw wastewater samples from 13 WWTPs distributed in an area of around 5600 square km. The sampling sites were tested between August 3<sup>rd</sup> and September 2<sup>nd</sup>, 2020. At the beginning of the surveillance period, the average *C. jejuni* load increased and this increase was somehow correlated by the authors to a *Campylobacter* outbreak that occurred in September in which three people consuming an Indian vegetable food were involved. However, more specific data were not collected, thus affecting the possibility of establishing a correlation with clinical data. The authors suggest carefully considering the essential role of key factors, such as analytical recovery efficiency and decay rate, to enhance the monitoring of *Campylobacter* load in sewage to eventually predict foodborne outbreaks (Zhang *et al.*, 2023).

The second retrieved study regarding *Campylobacter* was carried out by Kuhn *et al.* (2023) and is already described above. The data regarding *Campylobacter* show that during the study period, 1459 out of a total of 2616 cases of foodborne bacterial infections were due to *Campylobacter* (56%), for a total of six weeks with reported cases. Clinical cases and concentration of *Campylobacter* in wastewater showed a significant seasonal peak ( $p=0.26$ ). Moreover, cross-correlation time series analysis highlighted a correlation between *Campylobacter* load in wastewater and the number of clinical cases (correlation coefficient 0.51-0.58). In particular, for *Campylobacter*, the load increase in wastewater occurred two weeks before the registration of clinical cases. Unfortunately, in the study, the genetic correlation between the strains circulating in wastewater and those infecting humans was not investigated.

### ***Analytical methods to detect or quantify foodborne pathogens in wastewater***

Different analytical methods to detect and or quantify foodborne pathogens in wastewater were employed in the selected articles. (Hellmér *et al.*, 2014; La Rosa *et al.*, 2014; Kazama *et al.*, 2017; Kuhn *et al.*, 2023; Zhang *et al.*, 2023) primarily employed single or multiple qPCR, reverse transcription (RT)-qPCR, nested RT-PCR, or triplex qPCR. A couple of studies (Yan *et al.*, 2018; Yanagimoto *et al.*, 2020) used a culture-based method followed by qPCR and RT-PCR, either for pathogen detection or quantification, and in one study (Diemert and Yan, 2019) the authors performed whole-genome sequencing.

Regarding the method applied to concentrate pathogens in sewage samples, only three papers provided explicit information. In the study by Kuhn *et al.* (2023), pathogens in sewage were concentrated by means of centrifugation at 14.600 x g for 45 minutes at 4°C. Hellmér *et al.* (2014) concentrated viruses from wastewater adopting the adsorption to milk powder method, which involves the adsorption of viruses by flocculated milk protein particles that aggregate and settle by gravity. The aggregates dissolve in a phosphate buffer and are then ready to be detected by PCR after the nucleic acid extraction. The effectiveness of this method was also demonstrated by Calgua *et al.* (2018) in a study concerning the development of procedures to concentrate viruses from seawater. Hellmér *et al.* (2014) attempted to apply multiple qPCRs for detecting norovirus, astrovirus, rotavirus, adenovirus, Aichi virus, parechovirus, HAV, and hepatitis E virus simultaneously in the same test. However, they opted to employ single RT-qPCR since the sensitivity of the test for virus detection increased 10- to 100-fold in comparison with the sensitivity achieved with multiple qPCR. The authors found that qPCR can detect as few as 120 and 200 viral particles per milliliter of wastewater. Finally, Kazama *et al.* (2017) concentrated norovirus from 40 ml of wastewater sample to 1 ml of virus concentrate through polyethylene glycol precipitation. RNA was extracted with QIAamp Viral

RNA minikit. RT-qPCR was applied to determine the concentration of norovirus GI and GII in sewage samples. Norovirus amplicons were pyrosequenced. The application of pyrosequencing to detect norovirus in sewage is a recent approach that allows to identify multiple genotypes circulating in the human population and to comprehend their temporal variation.

Besides the papers selected in this review, mostly using PCR-based methods to either detect or quantify foodborne pathogens in wastewater, Tang *et al.* (2021) showed the suitability of shotgun metagenomic to detect pathogens, including foodborne pathogens, in wastewater. The authors tested water samples from a river and five discharge ports (one hospital, one food processing plant, one slaughterhouse, one pharmaceutical factory, one WWTP). Samples were tested by shotgun metagenomic to compare relevant pathogens and antibiotic resistance genes in the different sources. *S. enterica* (8.78%) and *Pseudomonas aeruginosa* (8.21%) have emerged as the most abundant pathogens in all tested samples. Additional pathogens identified in the tested samples were *Campylobacter*, *Listeria monocytogenes*, Shiga toxin-producing *Escherichia coli*, *Vibrio cholerae*, *Bacillus cereus*, and *Staphylococcus aureus*.

### ***Sampling methods to detect or quantify foodborne pathogens in wastewater***

The analysis of sewage begins with sample collection. The microbial communities present in wastewater can vary greatly across periods of hours to months, with considerable spatial variation within a specific network or between various geographical areas (Fierer *et al.*, 2022). The selected papers report two main types of sampling: grab and composite. Grab samples are taken at a single point in time, particularly at a time of the day that is considered “peak flow”, to maximize microorganisms load (Farkas *et al.*, 2018; Mejias-Molina *et al.*, 2023). On the contrary, a composite sample consists of a mix of several samples collected over the 24 hours of a single day, which is more indicative of the total population input, due to the temporal fluctuation of wastewater composition. Usually, composite sampling is done by autosamplers, which collect sewage samples at predetermined intervals (varying from minutes to hours) and then combine them to create a composite sample (Ahmed *et al.*, 2021; Mejias-Molina *et al.*, 2023).

Among the considered articles, two studies employed grab sampling methods (La Rosa *et al.*, 2014; Kazama *et al.*, 2017), four research involved the application of composite sampling techniques (Hellmér *et al.*, 2014; Yan *et al.*, 2018; Diemert and Yan, 2020; Kuhn *et al.*, 2023), while the others did not explicitly specify employed sampling methods (Yanagimoto *et al.*, 2020; Zhang *et al.*, 2023). The most detailed sampling protocol is that described by Yan *et al.* (2018). They collected raw sewage samples using a composite sampling approach articulated as 40 ml of sewage collected each hour. Their approach helped to average out short-term microorganisms’ fluctuations and provided a representative daily overview of the target microorganisms (Yan *et al.*, 2018). According to Ahmed *et al.* (2021), most studies used composite sampling since grab sampling can be inadequate for detecting low levels of microorganisms due to diurnal variations in their concentrations in wastewater. Composite sampling, on the other hand, is representative of a pool of samples collected at different hours and times of the day, and it is less sensitive to variations in pathogen concentrations within wastewater (Ahmed *et al.*, 2021). To date, there are no studies that have identified the gold standard sampling method for detecting foodborne pathogens in wastewater.

### **Conclusions**

In 2022, the number of foodborne outbreaks in Europe increased by 43.9% in comparison to 2021 and the number of deaths increased by 106.5% compared to 2021. These data emphasize the need for innovative surveillance methods for the monitoring and possibly prediction of foodborne outbreaks. Wastewater surveillance has proven to be highly effective during the COVID-19 pandemic. In contrast, this review shows a limited exploitation of wastewater surveillance to monitor foodborne pathogens. The little evidence available shows that the calculation of specific pathogen concentration thresholds in human wastewater, beyond which risk of foodborne outbreaks may emerge within a few



weeks, is a powerful tool for alerting authorities and triggering the adoption of tangible public health measures to mitigate the impact of foodborne outbreaks.

Following the approaches presented in the selected papers, we can state that an effective foodborne pathogen monitoring strategy can be articulated in three connected steps. The first includes testing wastewater with an untargeted method, such as shotgun metagenomic, to detect wastewater microorganisms belonging to different domains (Sala *et al.*, 2020; Tang *et al.*, 2021), including viruses and bacteria. The second consists of testing wastewater with a targeted method, *e.g.*, qPCR, to quantify those specific pathogens that, in the metagenomic dataset, display an increasing trend or exceed baseline concentration thresholds. The third involves the integrated wastewater and clinical data analysis and modeling to find meaningful epidemiological correlations or make predictions. Therefore, a close collaboration among public health practitioners, public institutions, researchers, and companies involved in wastewater treatment must be established and possibly supported by European policies.

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Online supplementary material

Supplementary Table 1. Results of the literature screening focusing on foodborne pathogens (both viruses and bacteria) and wastewater.