Sewer systems:
epidemiological imaging of populations

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Abstract

Three examples will be presented to demonstrate the capacity of sewage analyses to provide a unique understanding of the presence and spread of pathogens in the community, and to furnish data for planning intervention and measuring its success.

1. Geographical distribution of pathogens with multi drug resistant genes: Sewage analysis demonstrated their presence in the general population as well as in sewage catchment areas of inpatient institutes.

2. Correlation of the geographical distribution of Hepatitis A virus (HAV) in sewage with distribution of HAV clinical cases: HAV, presumed to have been brought under control in Israel through an intensive routine vaccination program, caused a small outbreak in 2012–2013. HAV was found in sewage samples collected from areas with and without reported clinical cases indicating a large area in which there were sub-clinical HAV infections.

3. The use of environmental surveillance for routine monitoring of poliovirus infections in populations: Israel has been polio-free since 1989. Routine environmental surveillance revealed introduction and subsequent sustained transmission of wild type one poliovirus (WPV1) in Israel in 2013 in the absence of poliomyelitis cases. Rapid expansion and modification of the program enabled the Ministry of Health (MOH) to obtain information regarding both the extent of penetration and the geographical spread of the WPV1 and to evaluate the efficacy of supplementary immunization activities (SIA).

Keywords: sewage monitoring, poliovirus, antibiotic resistant bacteria, hepatitis A virus.

1 Introduction

During the 19th century the risk from water borne diseases and the correlation between such risk and faecal contamination was well understood. Sewer systems were constructed first in cities all over Europe and later in the United States. Over the next 100 years the main surveillance efforts focused on monitoring the integrity and quality of wastewater systems as a way of preventing illness and environmental damage. During the second half of the 20th century, Melnick *et al.* demonstrated a correlation between the presence of viruses in sewage and the occurrence of cases in the community [1]. Greater understanding of the role of water in the transmission of infectious agents, and viruses in particular, led the World Health Organization (WHO) to recognize the importance of the problem [2].

The methods used to measure concentration and carry out detection in environmental microbiology – and in particular environmental virology – suffered from low efficiency and limited detection sensitivity [3]. Their improvement and the introduction of molecular methods together with the development of environmental epidemiology led to better understanding of the correlation between the distribution of pathogens in sewage systems and morbidity in the community.

This article demonstrates the strength of this current approach using three separate surveys conducted in Israel between 2012 and 2014:
1. Monitoring the presence of multi drug resistant bacteria in sewer systems and correlating their presence with catchment areas with and without inpatient institutions (INPI) such as hospitals and nursing homes.
2. Monitoring Hepatitis A virus (HAV) in wastewater during and after an outbreak of HAV in a highly immunized population in the greater Tel Aviv area.
3. Routine sewage monitoring of poliovirus (PV) as part of the certification of Israel as a poliovirus-free country and modification of the program in response to the presence of WPV1 in environmental samples in the absence of paralytic disease. Sewage surveillance provided information for identifying the introduction of WPV1, its sustained silent transmission, and the efficacy of supplementary interventional activities (SIAs).

2 Material and methods

2.1 Sewage samples

Sewage samples (10 L) were collected over 24 hrs at the entrance to sewage treatment facilities (STF) using in-line automatic composite samplers or at upstream branch points within the catchment areas of the STFs using automatic Sigma composite samplers (Sigma SD900 automatic samplers, HACH, CO, USA). The composite sample comprised a mixture of 24 or 48 individual samples gathered at timed intervals over the 24 hours period. For bacteriological tests, 1–2 ml of the composite sample was used directly. For virus detection, 1 litre was

concentrated to a final volume of 15–20 ml using the polyethylene precipitation methods as previously described [4].

2.2 Bacteriological tests

The pour plate technique for heterotrophic bacterial count [5] was used with minor modifications. 1–2 ml of raw sewage was added to 3 ml of melted selective Chromagar (for KPC, MRSA, plus VRE Following gentle mixing, the mixture were poured onto a 10 cm plate and incubated at $35 \pm 2°C$ for 48 hours.) and 3 ml of distilled water. Colonies suspected of being CRE (big blue or pink colonies), MRSA (pink colonies) or VRE (pink colonies) were isolated on Chromagar Orientation, Chromagar MRSA and Chromagar VRE respectively. The isolated colonies were gram stained and observed in a light microscope. Bacteria identification and determination of susceptibility to CRE and MRSA were performed using Phoenix™ System and MALDI-TOF. Uncertain identifications were tested classically according to the literature and by 16SRNA sequencing for confirmation. Identification and susceptibility of VRE were performed classically [6].

In order to determine whether the suspected colonies were indeed CRE, gene detection for bla_{KPC} /bla_{NDM-1} was performed using real time PCR (RT-PCR). DNA extraction was carried out on well isolated colonies and/or fresh sewage by NucliSENS® easyMAGTM or by EZ1 advanced [7].

As for MRSA and VRE, biochemical test were performed in order to determine whether the bacteria were Staphylococcus Aureus or Enterococcus sp. respectively. Susceptibility tests were performed to determine antibiotic resistance: cefoxitin for MRSA and vancomycin for VRE. If the bacteria were resistant, they were gram stained for final confirmation [6, 8].

2.3 Virological tests

2.3.1 Sewage samples processing
One litre of well-mixed sewage was treated and concentrated down to 20–30 ml [1] using polyethylene glycol (PEG) 6000, precipitated O/N at $4°C$ and spun down. The virus was extracted from the pellet using a PBS Tween 80, chloroform and 3% beef extract solution in 30 ml final volume for each sewage sample and kept at $4°C$ [4].

2.3.2 qRT-PCR assays
Total nucleic acid (NA) was extracted from well vortexed concentrated sewage samples using the NucliSENS® easyMAG™ system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Briefly, lysis was performed on 1 ml of concentrated sewage to inactivate the virus as recommended by the manufacturer. This was followed by NA extraction using the easyMAG™ extractor. Extracted NA was eluted in 55 μl elution buffer and stored at $-70°C$ pending analysis.

The HAV q-RT-PCR kit (genseg HAV – Primerdesign, UK) was used to detect HAV specific RNA.

The RNA from samples that tested positive for HAV and had a Ct < 30 (Ct – the qRT-PCR cycle at which the signal is first detectable above a threshold) was amplified by RT-PCR, using primers flanking the VP1-P2a region (~1100 nucleotides) as described by Costa-Mattioli *et al.* and sequenced for phylogenetic analysis [9].

A specific qRT-PCR assay for WPV1 was designed for rapid detection of WPV1 RNA extracted directly from concentrated sewage samples and from tissue culture supernatants [5]. Briefly, two sets of primers and probes were designed based on the VP1 sequences of 5 plaque purified WPV1 isolated from one of the first positive sewage samples. Molecular and phylogenetic characterizations based on complete VP1 capsid protein sequence for all virus isolates were performed as previously reported [10, 11].

3   Results

3.1 Multi drug resistant bacteria monitoring

Sixteen sewage sites within the greater Tel Aviv area were sampled. Table 1 illustrates the distribution bacteria with four different resistance genes at the sites that were monitored. All four resistance genes were detected with bla_{KPC} and VRE proving dominant (75% and 69% of sites, respectively), while bla_{NDM-1} and MRSA were present in fewer sites (13% and 19%, respectively). With the exception MRSA, similar results were observed for catchment areas of communities that had inpatient institutes (INPI) (9 sites) communities (7 sites) that didn't.

Table 1:   Distribution of four different resistance genes in communities with and without inpatient institutions.

	All sites positive/total (%)	Sites in INPI communities positive/total (%)	Sites in communities without INPI positive/total(%)
bla_{KPC}	12/16 (75%)	7/9 (78%)	5/7 (71%)
Bla_{NDM-1}	2/16 (13%)	1/9 (11%)	1/7 (14%)
MRSA	3/16 (19%)	3/9 (33%)	0/7 (0%)
VRE	11/16 (69%)	7/9 (78%)	4/7 (57%)

Analysis of the bla_{KPC} bacteria species in the sewage of the two types of communities revealed a marked difference. In the communities with INPI *Enterobacter cloacae* dominated (32.4%) while *Acinetobacter* sp. was not found.

In communities without INPI *Klebsiella pneumonia* dominated (50%) while *Citrobacter freundii* and *Enterobacter asburiae* were absent from the sewage samples.

Table 2: Percentage of bla$_{KPC}$ bacteria sp. found in sewage of catchment areas of communities with and without inpatient institutions.

bla$_{KPC}$ bacteria	Communities with INPI % positive sites	Communities without INPI % positive sites
Acinetobacter sp.	-	6.2
Citrobacter braakii	5.4	6.2
Citrobacter freundii	10.8	-
Enterobacter asburiae	2.7	-
Enterobacter cloacae	32.4	12.6
Eschericia coli	10.8	18.8
Klebsiella oxytoca	8.1	6.2
Klebsiella pneumoniae sp.	29.8	50

3.2 Hepatitis A virus surveillance

Following an outbreak of hepatitis caused by HAV, an environmental surveillance was conducted in the greater Tel Aviv region. Altogether 22 samples were collected. The outbreak started in March 2012 and over 80 cases were diagnosed by the end of the year. Eighteen sites were chosen for monitoring from catchment areas with cases and from adjacent areas without cases. The surveillance started in October 2012 and ended in February 2013. Fifteen of them tested positive for HAV. Figure 1 shows the regions that were sampled and the location of clusters of clinical cases. Almost all cases were in in the southern suburbs of Tel Aviv. A few cases were identified in the northern parts of the city and in Bat Yam and Holon, two satellite towns south of Tel Aviv. Sewage surveillance revealed an area with silently excreting people that was almost three folds greater than the one where most of the clinical cases were reported (big circles in figure 1).

We sequenced the VP1-P2a region of the HAV genome from RNA extracted from 12 patient's sera. All were 1B subtype. There were 2 distinct genomic sub-lineages: 1B-1, a majority (n=11) that were 97% homologous to a strain from Hungary (Gene Bank access number Ib/Hungary/E1F190998), and 1B-2 (n=1) that was 100% homologous to the same isolate. The sub lineages differed by 40 nucleotides ~ 4% of the area sequenced. The 1A sub-linage was found in all but one sewage sample and most of the patients (figure 2, patient 62 and site c115).

The 1B-2 linage was found in one patient and in one sampling site of a catchment area with no known epidemiological linkage (figure 2, patient 63 and site line B). Five sewage samples were collected during March, April and May 2013 from the main positive sites in Tel Aviv after the last clinical case had been reported. All tested HAV negative.
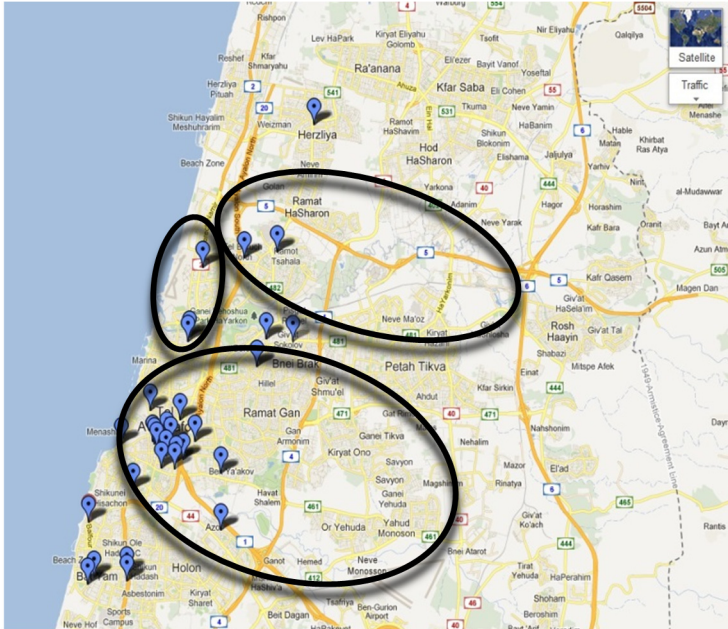
Figure 1: Greater Tel Aviv region map. The inverted tear drop signs mark the clusters of clinical HAV cases. The circles mark the zones of the HAV positive sewage sampling sites.

Patient 62(1B-1)

Patient 63(1B-2)

Sewer site line b(1B-2)

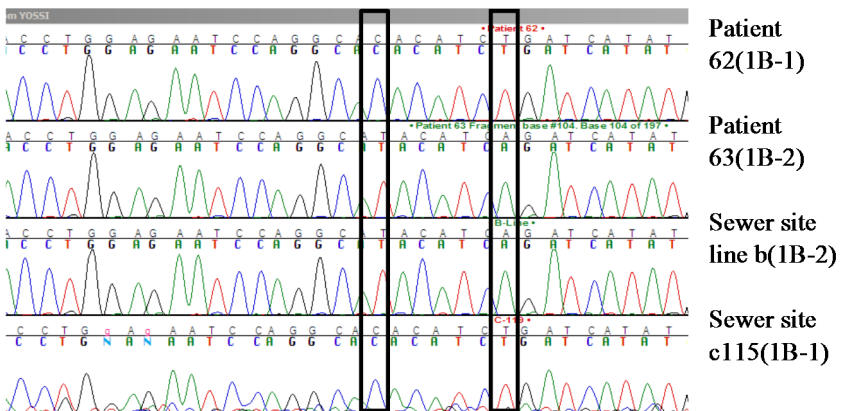Sewer site c115(1B-1)

Figure 2: Comparison between a partial HAV VP1-P2a sequence taken from two patients and samples from two sites in Tel Aviv.

In addition to this study, 13 sewage samples from various other major cities in Israel where there were no reported HAV cases, were analyzed at the time of the Tel Aviv outbreak. Five (38.4%) tested positive for HAV demonstrating widespread infections.

3.3 Polio virus monitoring

Between April 2013 (the collection date of the first sewage samples to contain WPV1 of South Asian origin (WPV1-SOAS)) and April 2014, we analyzed 486 sewage samples from surveillance sites throughout Israel for the presence of WPV1-SOAS (figures 3 and 4). An additional 7 samples dating backwards to Feb 2013 were analysed retrospectively. The routine monitoring protocol was modified to increase throughput, increase the number of surveillance sites, and decrease turn-around time for assay results. 176 of the 486 samples contained WPV1-SOAS. However, throughout this whole time there were no cases of paralytic poliomyelitis, thus the gold standard for polio surveillance for polio based on investigating all cases of paralysis for poliovirus infection failed to document this major event. This intensive surveillance program enabled the Ministry of Health to locate the epicentre of the virus (figure 3) and document its spread to central Israel (figure 4).

This knowledge also was used to base the Public Health Services response with supplementary immunization activities and to observe the effect of WPV1-SOAS. In addition to detecting the presence or absence of WPV1, we also measured the amounts of virus by plaque assay and by qRT-PCR. Figure 5 illustrates the usefulness of this additional level of semi-quantitative information for sewage collected at the mouth of the Rahat STF.

Figure 3: Monitoring for WPV1 in south Israel showing the epicentre of the virus's spread.
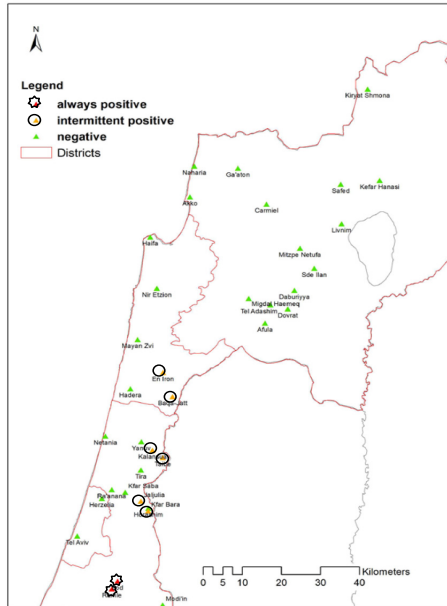
Figure 4:   Monitoring of WPV1 in central and northern Israel, and its spread to central Israel.
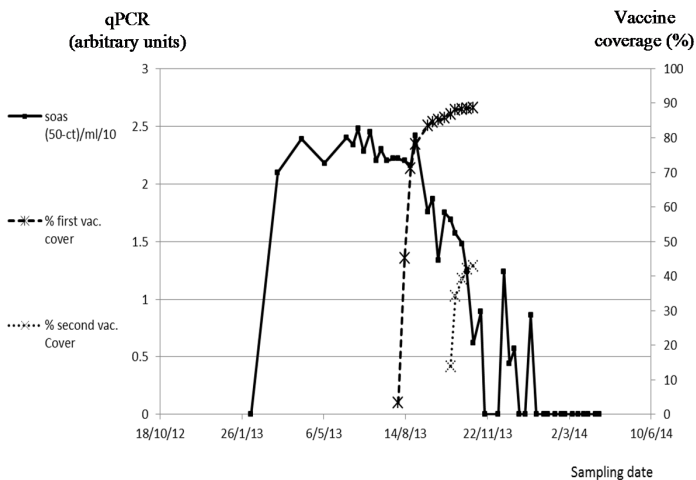

Figure 5:   Emergence and disappearance of WPV1 in the sewage of Rahat following two rounds of vaccination.

The graph shows the increase in virus in the sewage, a plateau and then the decline in the amount of WPV1-SOAS over time. Figure 5 also indicated the percent of children under ten years of age in Rahat who received one or two doses

of bOPV (bivalent oral poliovaccine containing Sabin 1 and Sabin 3 vaccine strains) and the time scale of when they received the vaccine. The immunization with bOPV shown in the figure was provided to children under 10 throughout the entire population of Israel, returning vaccine strains to sewage for the first time since oral polio vaccine was discontinued in 2005.

4   Discussion

This article highlights the utility of sewage surveillance as an important scientific approach, at times the only one, for the collection of important epidemiological data. The three examples discussed represent different sampling and identification strategies. Three decisions are crucial for optimal implementation of environmental surveillance for enteric pathogens. First, the scientific dilemma needs to be defined taking into account the fact that sewage samples comprise a huge mixture of individual faecal samples excreted by large populations. It follows that the pathogen, population of interest, and the selection of adequate sampling sites must be clearly identified. The second type of decision-making pertains to the sampling methods and the selection of adequate sampling sites from the engineering point of view. The choice of tools, teams and fieldwork all need to be evaluated. Third, decisions have to be made as to which of the available methods are most suitable for this type of research and what modifications may need to be made.

How was this applied to the three projects presented here?
In the first project we posed the following question: Are the bla_{KPC}, bla_{NDM-1} and the VRE genes present only in bacteria reported to be in patients residing in inpatient institutions in Israel [13] or are they, like the MRSA genes, carried by bacteria in the population at large? We identified sampling sites in catchment areas with and without inpatient institutions. We next arranged for the actual collection of samples. Finally, we modified the Total Heterotrophic Bacteria Count Test to make it a selective test for bacteria carrying the above-mentioned genes. Once we identified purified colonies of interest, conventional clinical methods were used.

Two important conclusions were reached from this study: First, antibiotic resistant genes, routinely identified in hospitals are also prevalent in the population at large. Second, the variety of bacteria species that host these genes in the population is slightly different from that found in areas with INPI. These results open up possibilities for using available advanced methods for achieving highly concentrated samples in order to monitor the presence of non-abundant newly introduced drug resistant pathogens before they cause disease.

In the second project we posed the question: Does the geographical distribution of HAV cases represent the true extent of HAV infections? The first challenge was to delineate the catchment areas where there were HAV cases and adjacent areas without cases. Correct choice of sampling sites revealed that the area with silently excreting people was much larger than expected. It also led to examination of sewage in other cities and the identification of HAV in some sewage samples where no cases had been reported at all. We concluded that the reported HAV cases in Israel, under conditions of a routine vaccination program, represent only

a small part of the larger true picture of infections, e.g. the clinical picture only partially represents the geographical spread of infections during outbreaks.

The third project illustrates population-based surveillance for poliovirus: Are there any individuals infected with poliovirus in the community? This project underlines the importance of flexibility in the conduct of environmental sewage surveillances. Different approaches and methods must be considered and adopted as appropriate if new challenges are to be faced. In the WPV type 1 event we had to design appropriate molecular tools for detection the first introduction of the SOAS strain of WPV1 into Israel [10]. As the virus continued to spread, we had to continuously select new sampling sites. In the final stages of the event, we had to design a reasonable and realistic long-term monitoring program to ensure the complete disappearance of the virus. As there were no clinical cases throughout the 14 months duration of the event, all decisions were based on results obtained from on-going environmental sewage sampling.

Methodological improvements in the field of environmental microbiology will enhance sewage surveillance as a primary epidemiological population-based tool. Development, validation and application of methods capable of efficiently concentrating microorganisms using technologies such as micro and ultra-filtration and new generation sequencing tools will expand possibilities for using sewage surveillance and will contribute to a better understanding of the epidemiology of enteric infectious diseases.

5   Conclusion

Traditionally transmission of gastrointestinal pathogens in Israel was evaluated solely through the instrumentality of clinical cases reports. Our experience emphasizes the importance of appropriate validated assays-based sewage surveillance, as a complementary approach. It will facilitates the better understanding the pathogens' epidemiology, will improve risk assessment and risk management, and will supply data for the formulation of public health related response. These advantages were demonstrated in different events namely, drug resistant bacteria monitoring, HAV outbreak in greater Tel Aviv, and WPV1 introduced into Israel.

In some cases (PV monitoring, new drug resistant genes) it is important to establish and maintain long term surveillance. It is the primary tool for detecting silent circulation of infectious agents before they reach clinical expression.

Acknowledgement

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