RESEARCH ON HUMAN VIRUSES IN DIFFUSE EFFLUENTS AND RELATED WATER ENVIRONMENTS

Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

BACKGROUND AND MOTIVATION

Waterborne diseases are the most important concern about the quality of water. According to authentic WHO estimates some 50 000 people die each day in the world due to water-related infectious diseases. Developing communities suffer most from these diseases. However, even in the USA an average of 38 epidemiologically confirmed waterborne disease outbreaks with some 100 000 cases are recorded each year, and indications are that the incidence is increasing. There is hardly meaningful epidemiologically confirmed evidence on waterborne diseases in South Africa. However, there is no reason to expect risks of infection to be any different from those in the rest of the world.

Viruses may account for up to 75% of infections associated with polluted water. Assessment of the virological safety of water is, therefore, of fundamental importance. Unfortunately viruses are not detectable by simple and inexpensive methods. In addition, commonly used indicators of faecal pollution such as coliform bacteria, have shortcomings for indicating the presence of viruses, or the efficiency of water treatment procedures with regard to viruses. The development of practical technology and expertise for assessment of the virological quality of water, and the removal of viruses in treatment and disinfection processes is, therefore, of major importance to the water industry.

A fundamental approach to the control of waterborne disease is to limit the pollution of water sources. This is particularly important with regard to viruses, because they may remain viable for weeks or months in water environments, and they are exceptionally resistant to water treatment processes. Also, the minimal infectious dose of viruses may be as low as a single viable particle. Information on sources of pollution is essential for the protection of water sources. There is, however, no information on the contribution of various waste water discharges to the contamination of water sources specifically with regard to viruses.

Against this background the objectives of the project may be summarised as:

1. Develop technology and expertise for research on human viruses in water, as well as the epidemiology of waterborne viral diseases.

2. Evaluate the contribution of waste water from informal settlements to the virological pollution of water sources.

OBJECTIVES

The primary objective was to study the impact of rapid urbanisation and informal settlements (ie, squatter communities) on the virological quality of water resources and supplies.

Representative urban or squatter situations were to be selected for investigating the incidence of human viruses and selected indicator organisms in diffuse effluents and water environments exposed to these effluents.

In order to supplement results of virological analyses and to better assess health risks, all possible information on related infections in the communities concerned would be obtained.

Since methods for the recovery and detection of the viruses in question still had many shortcomings, their ongoing refinement aimed at obtaining the best possible results was seen as an integral part of the study.

Anticipated benefits of the research results were seen to include new information on water pollution and related health risks which is essential for defining appropriate quality criteria. End users such as the Department of Water Affairs, the Department of National Health and Population Development, and local authorities, could apply the results in formulating management strategies for the protection and optimal utilisation of limited water resources and supplies, and for the control of waterborne diseases. At the same time the project was expected to make a major contribution to the establishment of advanced facilities and expertise, and the training of high level manpower. All of these were seen as essential to meet future challenges in the water industry and public health services.

SUMMARY OF MAJOR RESULTS AND CONCLUSIONS

Literature review

Literature on waterborne diseases has been reviewed in detail, and information relevant to this project has been assimilated and summarised for practical interpretation.

Technology development

Since infection may be caused by a single viable viral particle, both the quality monitoring of water supplies and research on waterborne viruses require sensitive and practical techniques for detection of the smallest possible number of viruses in water. Ongoing research in many laboratories worldwide focuses on this challenge. Efforts and progress accomplished as part of this project may be summarised as follows:

A practical and economic technique for the in-line and on-site recovery of viruses from water based on glass wool adsorption-elution filters has been developed and evaluated. The efficiency of recovery for a variety of human viruses compares favourably with that of procedures commonly used for this purpose. The inexpensive filters are convenient to use and can readily be forwarded to a laboratory for the recovery and detection of viruses. The procedure has been introduced for

the routine monitoring of the virological quality of raw and treated water supplies of Rand Water.

An affinity chromatography (AC) procedure for the selective recovery of specific viruses from water has been developed and evaluated. The technique is based on filter columns containing agar beads to which antibodies directed against the virus of choice have been attached. When water is passed through these columns, the antibodies trap the viruses concerned while other viruses and impurities pass through. The viruses can afterwards be released from the antibodies in concentrated and purified form detection. This method has valuable features for application in, for instance, studies on the role of water in specific disease outbreaks.

Technology for the isolation of viruses by propagation in cell cultures has been improved. In a detailed comparison of 8 cell culture systems, evidence has been presented that the most sensitive practical procedure requires the simultaneous inoculation of the following cell cultures: Primary vervet kidney cells, the BGM Green Monkey kidney cell line, and the PLC/PRF/5 human liver cell line. The susceptibility of each of these cell cultures for various viruses differs, but in combination at least any one of them supports the replication of a wide variety of viruses. This combination of cell cultures is now routinely being used in our laboratory for the virological analysis of water. We are not aware of a more sensitive cell culture system being used for the detection of viruses anywhere in the world. Evidence has also been presented that meaningful detection of viruses in water environments requires at least three passages, even in this sensitive combination of cell cultures. This is because many viruses are still infectious, but have been injured by prolonged exposure to unfavourable conditions in the water environment to the extent that they fail to immediately replicate in cell cultures and require some time for recovery.

In efforts to increase the sensitivity of cell cultures for the detection of viruses in water, efforts have been made to modify the susceptibility of cell cultures. Selected cell cultures were treated with certain chemicals known to affect viral infection under certain laboratory conditions. Treatment of cell cultures with 5-iodo-2'-deoxyuridine (IDU) increased the sensitivity to laboratory strains of poliovirus by up to 100-fold. In addition, the cytopathogenic effect caused by the virus in the cell culture was much clearer and detectable 2-4 days sooner than in untreated cell cultures. Treatment of cell cultures with various other chemicals, such as polyethylene-imine (PEI) also increased the sensitivity of some cell cultures to laboratory strains of viruses, although in most cases only by a factor which ranged from 2- to 10-fold. Initial findings indicate that the sensitivity of cell cultures to naturally occurring viruses is similarly increased, and both most probable number (MPN) assays and direct plaque assays on numbers of cytopathogenic enteric viruses in water environments yielded higher counts using treated cells. In addition, treatment of cells with IDU considerably facilitated the detection of cytopathogenic effects and reduced the time required for the detection of viral infection which has meaningful benefits.

Unfortunately many waterborne viruses are not detectable by conventional cell culture procedures as described above. This is because although viable they fail to produce a visible cytopathogenic effect, at least in cell cultures presently available and under laboratory conditions presently applied. These viruses are referred to as non-cytopathogenic. The detection of these viruses requires alternative methods. Overseas human volunteers have often been used in research on these viruses. Progress in work on the detection of non-cytopathogenic viruses carried out in this project may be summarised as follows:

Some non-cytopathogenic viruses infect cell cultures and get so far as to replicate their nucleic acid and some capsid proteins, but fail to produce complete viral particles or a visible cytopathogenic effect. Techniques have been established for detecting the nucleic acids and capsid proteins in cell

cultures of viruses which display this phenomenon. These viruses include astro, hepatitis A and rota. The advantage of this procedure compared to methods based on the detection of non-cytopathogenic viruses by direct detection of their nucleic acids or capsid proteins, is that it confirms the viability of viruses, and increases sensitivity because the quantity of nucleic acid and capsid protein is replicated, ie amplified, by the cell cultures.

Non-cytopathogenic viruses may be detected by electron microscopy (EM). Unfortunately, however, this method requires sophisticated facilities and expertise, cannot distinguish between viable and non-viable viruses, and conventional procedures cannot detect less than about 1 000 000 particles per ml. In addition, conventional EM cannot distinguish between many morphologically similar viruses, such as coxsackie, polio, echo and hepatitis A viruses. In this project modifications have been established in which specific antibodies are used to clump viruses for increased detection sensitivity (a procedure known as immune electron microscopy), and to facilitate distinction between morphologically similar viruses. This procedure is now routinely being used in the typing of viruses isolated from water. It has also been used to study for the first time in South Africa the role of potentially waterborne calici- and astroviruses in cases and outbreaks of gastroenteritis.

Non-cytopathogenic viruses may be detected by procedures in which antibodies specific for capsid proteins (antigens) are used. Attachment of the antibodies to the viruses is detected by labelling the antibodies with enzymes which catalyse a colour reaction (enzyme-linked immunosorbent assays, ELISA) or other markers such as radio-isotopes (radio-immuno assays, RIA). The level of sensitivity of these immuno-assays is about the same as that of EM, and like EM they cannot distinguish between viable and non-viable viruses. Although not sensitive enough for meaningful monitoring of water quality or assessment of the efficiency of treatment processes, these methods are ideally suited for the detection of non-cytopathogenic viruses in the stool of infected individuals. In this project immuno-assays for various viruses have been established and used in research on infections in individuals exposed to polluted water. These techniques were, for instance, used in the first research in South Africa on infections by gastroenteritis viruses such as the Norwalk virus, other caliciviruses and astroviruses in canoeists exposed to sewage polluted river water, and in consumers of sewage-polluted drinking water.

The above immuno-assays can be carried out in reverse, ie, using viral antigens to detect the presence of antibodies directed against certain viruses in the blood (serum) of people. Specific antibodies are produced in response to viral infections. This implies that the presence of antibodies directed against a certain virus implies that the person has been infected by that virus. The detection of specific antibodies is routinely used for the laboratory diagnosis of viral infections. The method is particularly valuable in the diagnosis of infection by non-cytopathogenic viruses. This implies that infections can be confirmed without ever detecting the causative virus itself. This has the further implication that the presence of antibodies in people offers fail-safe evidence of the presence of the virus. In this project assays for a number of potentially important waterborne viruses have been established and optimised. These methods have, for instance, been used to submit the first evidence that the hepatitis E virus (HEV) occurs in South Africa. The HEV is notorious for waterborne outbreaks in some parts of the world. For instance, in 1991 a sewage-polluted drinking water supply caused an outbreak of hepatitis E with some 79 000 cases in Kanpur, India. Hepatitis E is clinically similar to the well-known hepatitis A, and both are typically waterborne, but the viruses which cause these diseases are clearly distinguishable and belong to different families of viruses. In this project surveys for antibodies (seroprevalence studies) have also been used to monitor infections by other non-cytopathogenic viruses such as calici and astro gastroenteritis viruses in canoeists exposed to polluted river water, people who consumed sewage-polluted drinking water, and individuals involved in outbreaks of gastroenteritis caused by

potentially waterborne viruses.

The above methods have also been incorporated in a refined protocol for the routine typing of viruses isolated from water environments. This protocol includes cytopathogenic effects in cell cultures, the inoculation of newborn mice, cell culture neutralisation assays, investigation of inclusion bodies in stained cells, electron microscopy, and immunological tests.

The approach to the detection of non-cytopathogenic viruses which is world-wide rapidly gaining ground is based on the detection of the nucleic acid of viruses by means of molecular techniques such as the polymerase chain reaction (PCR). These detection techniques are extremely specific, the sensitivity may be superior even to that of cell culture propagation, and results are usually available considerably sooner than those of cell culture propagation. Disadvantages are that at this stage these methods are generally speaking still in a stage of development and require sophisticated facilities and expertise. However, rapid progress is being made in the simplification of the procedures and commercial test kits are already available for some viruses such as the AIDS-virus. An important disadvantage of these techniques is that they cannot distinguish between viable and non-viable viruses. However, they still have major benefits for water quality assessment. For instance, the absence of viruses from water, "dead or alive" implies that the water is safe. Positive results would imply a potential risk because some of the viruses may be viable, and the presence of these viruses also indicates the potential presence of other viable viruses. The extreme specificity of these methods is also used to type viruses. In this project PCR procedures have been established for the detection of a number of non-cytopathogenic viruses, including calici, astro, hepatitis A and E, enteric adeno, and rota. Results of the first comparative assays also indicate that our PCR procedures for cytopathogenic viruses are more sensitive and faster than cell culture propagation, and the experimental application of PCR procedures for the routine monitoring of enteroviruses in water supplies is in progress.

Epidemiological studies

Two outbreaks of gastroenteritis have been studied. The causative agents have been identified as certain types of caliciviruses. These were the first recordings of involvement of the viruses concerned in outbreaks of gastroenteritis in South Africa. The same or closely related viruses are known as common causes of gastroenteritis in at least some parts of the world, and their transmission is typically associated with water and food. Although contaminated food was eventually identified as the most likely source of infection in both outbreaks studied as part of this project, the investigations offered a valuable opportunity to evaluate expertise for research on disease outbreaks associated with contaminated water, and to identify the aetiological agent.

Epidemiological studies were carried out on participants in the Dusi Canoe Marathon which is each year in January contested down the Umsindusi and Umgeni Rivers from Pietermaritzburg to Durban. The water in these rivers is exposed to sewage pollution, and at least at times counts of faecal coliform bacteria and coliphages exceeded levels generally accepted as safe for water intended for direct contact recreation. Analysis of data obtained by questionnaires indicated a significantly higher incidence of clinical complaints potentially related to infections by waterborne viruses among participants with extensive exposure to the river water than among accompanying persons with limited or no exposure to the water. However, serological analysis of blood specimens from 577 canoeists failed to indicate a higher incidence of infection by Norwalk and hepatitis A and E viruses than in non-canoeists. The tests only covered a small number of viruses typically associated with exposure to polluted water. Explanation of clinical complaints among

canoeists, and assessment of the theoretical risk of infection associated with recreational exposure to sewage polluted water would require more comprehensive analyses. Blood serology indicated a meaningfully higher incidence among canoeists only for schistosomiasis infection.

In August 1994 data were gathered on a typical waterborne outbreak of gastroenteritis in a suburb of Knysna. Gastroenteritis viruses were detected in stool specimens of clinically ill patients. Enteric viruses were isolated from the drinking water supply. The water also contained levels of faecal coliforms, enterococci, and somatic and male-specific coliphages in excess of limits recommended for drinking water. Faecal pollution was ascribed to an error in the drinking water supply system (unpublished data).

Astroviruses were detected in about 3% of more than 1000 stool specimens from gastroenteritis patients, predominantly children. This indicates that the incidence of these viruses typically associated with waterborne transmission is similar to that in many other parts of the world, which implies that the risk of waterborne transmission may be similar to that in countries where waterborne transmission is on record.

In another study analysis of more than 1000 stool specimens from gastroenteritis patients, including hospitalised children and adults, some of which cases from two outbreaks, indicated that the incidence in South Africa of caliciviruses such as Norwalk virus, was similar to that in other parts of the world where these viruses are typically associated with waterborne transmission.

A seroprevalence study on 555 Dusi canoeists exposed to polluted river water and 227 Pretoria students with no meaningful exposure to sewage polluted water, revealed an overall 2,05% incidence of antibodies to the hepatitis E virus. This was the first meaningful evidence of the presence of the hepatitis E virus in South Africa. These findings have since been confirmed by others who reported the presence of hepatitis E antibodies in up to 14% of individuals in certain high risk communities. Apart from one retrospective study in which serum analyses indicated that clinical hepatitis in certain patients was probably caused by the hepatitis E virus, there is no record of clinical cases of infection contracted in the country. The absence or rare incidence of clinical cases of hepatitis E remains to be explained. Important, however, is that there is sound evidence of the virus being present in the country, which implies that the possibility of waterborne hepatitis E outbreaks similar to those in certain other parts of the world cannot be ignored.

Studies on diffuse effluents

Research on the quality of diffuse effluents from informal settlements with restricted sanitary services focused on two sites, namely Botshabelo near Bloemfontein, and Stanza Bopape Village in Mamelodi, Pretoria. At both sites representative samples of effluents and related water environments were collected over a period of one year in order to cover all seasons. Analysis of the samples, characterisation of faecal indicators, typing of viral isolates, and processing of results took much longer. Final results and conclusions may be summarised as follows:

The microbiological quality of diffuse effluents from informal settlements in Botshabelo, and the impact of these effluents on the quality of receiving waters has been studied extensively during 1993. The effluents contained high counts of faecal bacteria and phages. The highest counts were recorded in storm water run-off. This run-off had faecal coliform counts as high as 4 400 000 per 100 ml, which is in the same order as many sewage effluents. Counts of faecal indicators were much lower in similar storm water run-off from a typical Bloemfontein suburb with well established

sanitary services. Storm water run-off from a Bloemfontein suburb with restricted sanitary services had levels of faecal indicators resembling those of Botshabelo storm water run-off. Effluents from Botshabelo primarily drain into the Klein Modder River, which drains into the Modder River. At least some residents in the area have contact with the water in the Klein Modder and Modder Rivers, and may even utilise the water for recreation or domestic purposes. The Modder River feeds the Mocke's Dam, a raw water source for the City of Bloemfontein and various other communities. Levels of faecal indicators were considerably higher in the Modder River downstream from Botshabelo than upstream, and in a number of streams in the area not exposed to effluents from Botshabelo. Elevated levels of faecal indicators were detectable in the Modder River up to 20 km downstream from Botshabelo. Counts of faecal indicators in the Klein Modder River were lowest during the dry season. However, some faecal indicators were present at all times, possibly as a result of seepage from pit latrines. Indicators of human faecal pollution were detected in water of the Mocke's Dam, but levels were within limits considered acceptable for recreational use of the water. No viruses were detected in representative samples from effluents and river water. The failure to detect viruses may be due to a number of reasons, which have not been investigated in detail. A battery of indicator organisms for distinction between faecal pollution of human and animal origin has been evaluated and established as part of this study. One reason was a relatively high density of domestic animals (eg cattle and dogs) in the study area. There is also a need for technology and expertise to distinguish between faecal pollution of human and animal origin in many other situations.

The impact of diffuse effluents from Stanza Bopape Village on the Edendale Spruit which runs through this informal settlement has been studied during 1994. This stream drains into the Pienaars River which feeds the Pienaars River Dam. The water in the river and dam is being utilised for irrigation, recreation and possibly even for domestic purposes by some residents in the area. A total of 210 representative water samples collected at regular intervals has been analysed. For purposes of comparison samples from sewage in the adjacently located Mamelodi with a sewerage system were included. Average faecal coliform counts per 100 ml in the Edendale Spruit were 800 upstream and 1 544 900 downstream of Stanza Bopape Village. Counts of other indicators of faecal pollution showed similar increased downstream of Stanza Bopape Village. After showers of rain when storm water from Stanza Bopape Village drained into the Edendale Spruit, counts of faecal indicators were exceptionally high in the Spruit. Viruses were never isolated from samples of Edendale Spruit water upstream, but from 70% of samples downstream of Stanza Bopape Village. Viruses were isolated from 95% of Mamelodi sewage samples. A total of almost 500 viruses has been isolated and typed. Counts were not directly compared to those in other sewage effluents, but compared to earlier data, counts of viruses and faecal indicators in Mamelodi sewage were exceptionally high. Subsequent quantitative tests on samples of the waste water yielded counts of cytopathogenic viruses as high as 800 000 per litre, which exceeds the highest counts on record in the literature. Typing of the viral isolates yielded valuable information on viruses prevailing in a typical faecally polluted water environment, as well as in the communities concerned. For instance, a high incidence of vaccine strains of polioviruses in the absence of wild strains indicates success in immunisation campaigns and a low risk for poliomyelitis infection. The most common isolates were coxsackie B viruses. This is the most comprehensive investigation of viruses in a natural water environment carried out to date in South Africa. As far as we know it is also the most comprehensive study on record for viruses in waste water from an informal settlement anywhere in the world. Data on enteric infections in residents were obtained from clinics serving the area. Analysis of stool specimens from 134 gastroenteritis patients in the Stanza Bopape Mamelodi area indicated infections by viruses including rota, enteric adeno, calici, astro and small round viruses. These viruses are typically associated with waterborne transmission. However, it was not possible to relate the infections to any particular water supply, and it was also not possible

to recover the same viruses from sewage or the Edendale Spruit. Such epidemiological confirmation of waterborne disease would require more detailed investigations.

ACHIEVEMENT OF OBJECTIVES

A user-friendly glass wool adsorption-elution procedure for the economic, simple, in-line and on-site recovery of viruses from water has been successfully introduced for water quality monitoring. This system has considerably reduced the cost and convenience of routine virological water quality monitoring. The affinity chromatography procedure for the selective recovery of viruses of choice from water is ready for application in studies on for instance epidemiological investigation of waterborne outbreaks and research on specific viruses.

The establishment of a combination of cell cultures and protocol for the sensitive and reliable isolation of cytopathogenic viruses is an important contribution to technology and expertise for water quality monitoring and research on waterborne viruses.

In the past the cost of analysing a water sample for viruses using cell culture detection was about 10 to 100 times more than that of a test for faecal coliform bacteria. These virus tests can now be offered at about 5 to 10 times the cost of faecal coliform tests. Unfortunately the tests still require relatively sophisticated facilities and expertise.

Progress in the development of methods for viruses not detectable by cell cultures now offers opportunities to address many viruses for the first time in research and water quality assessment in South Africa. These viruses include hepatitis A and E, calici, rota, astro and adeno, which are most commonly associated with waterborne transmission. This work facilitated the first inclusion of molecular techniques as an integral part in routine water quality monitoring in South Africa.

The molecular techniques presently available have potential for substantial further development and improvement with regard to sensitivity, reliability and the spectrum of viruses detectable. At this stage the techniques still require relatively advanced laboratory facilities and skills. However, there is reason to believe that these tests can eventually be simplified and that the cost can be reduced to levels within reach of many laboratories. PCR test are already available in the form of relatively simple and inexpensive commercial kits for detecting viruses such as the AIDS virus in patient serum.

Studies on outbreaks of diseases caused by enteric viruses offered an opportunity to optimise technology and expertise for research on waterborne diseases. The results yielded the first evidence of the presence in South Africa of viruses which are known to have caused waterborne disease outbreaks in other parts of the world. Of special interest in this regard is the first evidence that the hepatitis E virus (HEV) may be endemic in South Africa. This calls for further research because HEV frequently causes waterborne hepatitis outbreaks in certain parts of the world. It is not clear why such outbreaks apparently have not yet occurred in South Africa. There is no reason to believe that such outbreaks cannot occur in the country.

The outbreak of waterborne viral disease at Knysna is typical of outbreaks on record for many parts of the world. This incident illustrates the vulnerability of drinking water supply systems to contamination and underlines the importance of reliable water quality monitoring.

The two study areas selected for assessment of the impact of diffuse effluents from informal settlements on water sources were according to all indications typical examples of similar situations in many other parts of the country. Effluents at both sites contained exceptionally high counts of faecal bacteria and phages, and these effluents substantially increased the levels of faecal pollution of receiving waters. Indirectly related information has also been obtained from the Dusi Canoe Marathon with recreational exposure to river water subject to faecal pollution from sources including diffuse effluents from informal settlements. The results of all these investigation show that diffuse effluents from informal settlements with restricted or no sanitation carry heavy loads of faecal organisms, and in at least some cases, also large numbers of viruses. Diffuse effluents from informal settlements may, therefore, constitute a major source of faecal pollution, including heavy loads of viruses, to water sources such as receiving streams, rivers and impoundments. These observations call for special attention to sanitation at informal settlements with due respect to the following considerations:

1. The pollution of receiving waters constitutes a health risk to people who directly use the water for recreation, washing, irrigation, and domestic purposes, as often happens in developing communities.
2. The public health implications of the risk of infection constituted by this pollution of water sources should not be underestimated. Many of the viruses and other pathogens concerned may have a low mortality rate and the consequences may largely be limited to socio-economic disease implications. However, this pollution of water sources also offers ideal opportunities for the rapid spread of more serious diseases such as typhoid fever, cholera, poliomyelitis and hepatitis E. Once a case of these diseases occurs in a community and the pathogens infiltrate water sources it becomes extremely difficult if not impossible to prevent outbreaks or epidemics with potentially disastrous consequences.
3. The pollution of water sources has technical and financial implications for utilisation of the water for the production of drinking water because advanced and expensive treatment and disinfection is required to produce safe drinking water from polluted raw sources.
4. The data on viruses in this study are limited to cytopathogenic viruses detectable by cell culture. These viruses probably represent the tip of the iceberg of waterborne viruses, excluding virtually all gastroenteritis and hepatitis viruses, which are most commonly involved in waterborne disease, but not detectable by cell culture propagation.
5. This study did not address the impact of diffuse effluents from informal settlements on ground water sources. Theoretically high density low socio-economic informal settlements with inadequate sanitation may have a far reaching impact on the quality and fitness for human consumption of ground water.

In addition to the above, the project has made a major contribution to capacity building and the training of manpower, which included two PhD students, three MSc students, a number of BSc (Hons) students, and a number of technologists and research assistants. This manpower in a specialised field of expertise is essential for addressing future challenges in water quality control and for supply of safe water supplies to all people in our country.

RECOMMENDATIONS FOR FURTHER RESEARCH AND TECHNOLOGY TRANSFER

Technology developed and established in this project offers attractive opportunities for substantial further improvement. This refers in particular to molecular techniques alone and in combination with cell culture amplification for the detection of viruses not detectable by conventional procedures.

Theoretically it should be possible to substantially increase the sensitivity of molecular techniques for detecting low numbers of viruses in water environments.

There is reason to believe that molecular techniques can eventually be developed into relatively simple and inexpensive kits within reach of many laboratories for routine quality monitoring of water supplies.

The finding that the hepatitis E virus (HEV) may be endemic in the country should be followed up. It is important to know why cases or outbreaks of hepatitis E are not seen in the country. This seemingly unusual epidemiology of a typically waterborne virus should be clarified, and the possible role of water in the spread of the virus in South Africa should be investigated in order to prevent outbreaks and epidemics similar to those on record in some other parts of the world.

Technology and expertise now available offer opportunities for more detailed research on waterborne diseases in the country, in order to get a better understanding of the health impact, the waters involved, the pathogens concerned, and the mode of waterborne transmission. For instance, according to our information the Medical Research Council has identified water as a major cause of intestinal infections in certain rural communities in the North-West Province. However, there would not seem to be meaningful data on the aetiological agents of the intestinal infections, and the microbiological quality of the water concerned, neither would there seem to be details on whether the infections were associated with ground water, surface water, piped water, or safe water contaminated in the household. Information along these lines is required for strategies aimed at controlling waterborne diseases.

The findings in this project that wastes from high density low income informal settlements may heavily contaminate surface water sources, calls for an investigation of the impact of wastes from these settlements on the quality of ground water sources. In the absence of efficient sewerage systems human excreta may reach ground water through seepage from pit latrines, French drains and even from the surface during rain.

This project and data in the literature outline the need for details on the microbiological quality of water sources and supplies. This includes details on the efficiency of water treatment systems, and the survival of pathogens in water environments. This information is required for the optimal utilisation of limited water resources, and the supply of safe water.

MAJOR RESEARCH OUTPUTS

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Marx F E: Detection of human astroviruses in South Africa. PhD (Medical Virology), University of Pretoria. In progress.

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OBJECTIVES

See Executive Summary

MOTIVATION

The potential of water to transmit pathogenic micro-organisms is one of the most important concerns in the water industry. Some time ago the World Health Organization has estimated that each year the health of some 500 million people is adversely affected by shortcomings in sanitation and water supply, and that about 10 to 20 million of these people die. Even in the USA an average of 38 waterborne disease outbreaks with some 100 000 cases is recorded each year. Indications are that the incidence of these infections is increasing.

Viruses feature prominently among the wide variety of pathogens transmitted by water. Available data indicate that in the USA more than 60% of waterborne diseases are caused by viruses. Illnesses associated with polluted bathing beaches and other recreational waters, and the consumption of contaminated seafoods, are almost exclusively caused by viruses. Viruses are readily transmitted by water because they are excreted in large numbers by infected persons. These viruses are released into water environments via waste water discharges, where they may remain viable for months because they are exceptionally resistant to unfavourable conditions. Water or food contaminated with these viruses constitutes a high risk of infection because the minimal infectious dose of the viruses may be as low as a single viral particle.

Acute viral infections associated with polluted water include hepatitis A and E viruses, polio, coxsackie and other enteroviruses, adenovirus types 40 and 41, and rota, calici, astro and corona viruses, as well as a wide variety of unclassified gastro-enteritis viruses known as "small round viruses". Human papilloma and other viruses associated with non-acute long-term effects including cancer, may also be transmitted by water.

Virtually no information is available on the transmission of diseases by water in South Africa, primarily because it has never been investigated in meaningful detail. This lack of important information creates a false sense of security in a situation which is actually favourable for waterborne diseases. Factors which play a role in this regard include limited natural water resources, inconsistent rainfall, rapid population growth and a large component of low-income communities.

At the time of the intended study the risk of waterborne disease was aggravated by socio-political and economic developments. These included the enhanced movement of people, and rapid third-world urbanisation and squatting under socio-political pressures which have little understanding of the importance of appropriate sanitation, the protection of water resources, and the safety of water supplies. The declining economic situation limited remedial actions.

The objective of the study was, therefore, to select representative urban and squatter situations for investigating the incidence of human viruses and related indicator organisms in diffuse effluents and water environments affected by these effluents. Although the study was primarily aimed at human viruses, it would inevitably also render valuable information on other waterborne pathogens. In order to augment information on water quality, and to better assess related health risks, all possible information on the incidence of related diseases in the communities concerned was to be gathered.

Progress in the establishment of facilities and expertise for research on viruses in water had reached the stage where the project could be attempted with confidence. However, there was scope for further refinement of existing techniques and the development of methods for many viruses which were not detectable at the time. Ongoing technology development was, therefore, seen as an integral part of the project in order to obtain the best possible results. This technology was, of course, also required for other purposes such as assessment of the efficiency of water treatment and disinfection processes.

A substantial part of the work was due to be carried out by post-graduate students, which implied that the project would make a valuable contribution towards the training of manpower in a sophisticated field of water quality assessment.

COLLABORATION

Dr W David Cubitt of the Hospital for Sick Children in London visited the Department during April and May 1992. Dr Cubitt is internationally recognised as leader in the field of research on enteric viruses. He assisted in the establishment of new techniques for the detection of various viruses and their antibodies, and supplied most valuable reference viruses, antibodies and reagents.

Dr Christine L Moe of the Gastroenteritis Unit, Centers for Disease Control and Prevention, Atlanta, Georgia, USA, visited the Department in July and August 1993. She made valuable contributions to our research on gastroenteritis viruses, notably the Norwalk and related viruses.

Prof Xi Jiang of the Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, Virginia, USA, visited the Department in June and July 1994. Prof Jiang is an international expert on the detection of viruses by molecular techniques and he made a major contribution to the development of technology along these lines in our laboratory.

Collaboration with these experts continued after the visits.

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NOTE

In view of the massive amount of paper on which work related to this project has been recorded, this report only consists of a summary of achievements, and a few key papers and reports in a series of selected appendices. Further details on specific aspects are available in publications, theses, conference presentations and reports (see Research Outputs), which are available in published literature or on request from the corresponding author or the Water Research Commission.

1. LITERATURE REVIEW

The general literature review (Grabow, 1996) (Appendix 1) outlines the importance of the field of research, and research needs and priorities. Details of various technical aspects have been reviewed in the publications and reports concerned.

2. RESEARCH ON VIROLOGICAL TECHNIQUES

2.1. Recovery of viruses from water

2.1.1. Glass wool filtration

A practical and economic technique for the in-line and on-site recovery of viruses from water based on glass wool adsorption-elution filters has been developed and evaluated. Evidence has been presented that the method has a relatively high efficiency of recovery for a variety of cytopathogenic viruses, and that it also recovers viruses such as Norwalk and astro. The technique proved superior to commonly used methods, and is now routinely used in our laboratory. The procedure has been described (Grabow and Taylor, 1993) (Appendix 2), and further details are due to be published.

2.1.2. Affinity chromatography

A comprehensive study on affinity chromatography (AC) has been completed (Potgieter, 1995). Test water is passed through a column which contains agar beads to which specific antibodies are linked which trap viruses against which the antibodies are directed. Viruses of choice can, therefore, selectively be recovered from water. The glass wool adsorption-elution method recovers all viruses non-selectively from water. The efficiency of recovery (EOR) of AC was more than 95%, but most viruses were inactivated in the process. However, inactivated viruses are still detectable by molecular techniques and electron microscopy, which implies that the method has attractive features for certain purposes. Basic findings have been described (Potgieter *et al*, 1995) (Appendix 3).

2.2. Detection of viruses

2.2.1. Cell culture detection

2.2.1. Selection of cell cultures

The efficiency of a variety of cell cultures which may be considered for the detection of cytopathogenic viruses has been compared (Potgieter, 1995; Grabow, 1996) (Appendix 3). Best results were obtained by using simultaneously primary vervet kidney cells, the BGM vervet kidney cell line and the PLC/PRF/5 human liver cell line. In tests on environmental waters, viruses were often only detected after three blind passages in these cells. The findings have been summarised (Potgieter and Grabow, 1995) (Appendix 3).

2.2.2. Modification of cell culture susceptibility

Treatment of a number of cell cultures with various chemicals considerably increased their susceptibility to a variety of laboratory strains of cytopathogenic enteric viruses. This treatment unfortunately failed to likewise increase the susceptibility of the cells to naturally occurring viruses in wastewater. However, even in this case cytopathogenicity was visible 2-4 days sooner, and the cytopathogenic effect was much clearer. This reduces the time required for virus testing, and results are unmistakable, which has important practical implications for virus testing. A detailed thesis is in preparation (Botma, 1996), and results have been summarised (Botma and Grabow, 1995) (Appendix 3).

2.2.3. *In situ* cell culture detection

Sensitive techniques for the detection of viruses by amplification of viral antigens or their nucleic acid in cell cultures have successfully been developed for astroviruses (Schildhauer *et al*, 1992; Marx *et al*, 1995), hepatitis A virus (Schildhauer *et al*, 1992) and rotaviruses (Schwalb and Grabow, 1995) (Appendix 3).

2.2.2. Electron microscopy

A practical method for the characterisation of polio and coxsackie viruses has been developed (Appendix 4). This method proved of major value in the typing of viral isolates described in Appendix 6.

2.2.3. Enzyme immuno-assays

Enzyme immuno-assays have been established for various viruses, including hepatitis E and Norwalk viruses. Techniques are available for the detection of viruses and their antibodies, and made it possible to conduct the first studies on the incidence of these typically waterborne viruses in South Africa (Grabow *et al,* 1994; Taylor *et al*, 1995).

2.2.4. Typing of virus isolates

Routine procedures have been established for the typing of viruses isolated from water environments. These procedures include cytopathogenic effects in cell cultures, the inoculation of newborn mice, cell culture neutralisation assays, investigation of inclusion bodies in stained cells, and electron microscopy. Isolates difficult to type are submitted to the National Institute for Virology for assistance, which includes the molecular distinction between vaccine and wild-type strains of polioviruses.

2.2.5. Molecular techniques

Details on molecular techniques which have been established for the detection of viruses not detectable by conventional methods have been described in detail. These include methods for small round structured viruses (SRSVs) including the Norwalk and related viruses (Wolfaardt *et al*, 1995), hepatitis E virus (Appendix 5), astrovirus (Marx *et al*, 1995), enteroviruses including non-cytopathogenic types (Wolfaardt and Grabow, 1993, Appendix 3), hepatitis A virus (Taylor and Wolfaardt, 1995, Appendix 3), rotavirus (Schwalb and Grabow, 1994, Appendix 3). Publications with further details on work regarding some of these viruses, including rotavirus, are in preparation. These techniques opened doors to the first meaningful studies in South Africa on the incidence in water of viruses which are not detectable by conventional methods. This makes it possible to study waterborne viruses in new perspective (Grabow, 1995, Appendix 1).

2.3. Application of techniques in practice

The above techniques have successfully been applied in practice for various research purposes described in detail elsewhere. See list of publications, conference presentations and theses under "Research Outputs", Appendices, and reports on epidemiological studies and studies on diffuse effluents below. This work includes the following:

2.3.1. Introduction of glass wool adsorption columns for routine monitoring of the virological quality of water supplies (Grabow and Taylor, 1993).

2.3.2. First establishment in South Africa of molecular techniques for the detection of typically waterborne viruses such as small round structured viruses (SRSVs) including Norwalk virus (Wolfaardt *et al*, 1995), hepatitis E virus (Marx and Grabow, Appendix 5), astrovirus

(Marx *et al*, 1995), enteroviruses including non-cytopathogenic types (Wolfaardt and Grabow, 1993, Appendix 3), and rotavirus with *in situ* amplification (Schwalb and Grabow, 1994, Appendix 3).

2.3.3. First reports in South Africa on outbreaks of gastroenteritis caused by viruses typically associated with waterborne transmission, ie small round structured viruses (SRSVs) including Norwalk virus (Taylor *et al*, 1993; Wolfaardt *et al*, 1995).

2.3.4. First molecular confirmation of gastroenteritis infections in South Africa caused by SRSVs and astroviruses, and molecular characterisation of the strains involved (Taylor *et al*, 1993, 1995; Marx *et al*, 1995; Wolfaardt *et al*, 1995).

2.3.5. First meaningful data on viral infections associated with recreational exposure to river water in South Africa (Grabow *et al*, 1994; Taylor *et al*, 1995).

2.3.6. First evidence of the presence of the hepatitis E virus in South Africa (Grabow *et al*, 1996, Appendix 6).

2.3.7. First studies on viruses and faecal indicators in diffuse effluents from informal settlements (Jagals, 1994; Jagals *et al*, 1995; Appendix 7).

2.3.8. First detailed study in South Africa on the characterisation of cytopathogenic viruses in wastewater (Appendix 7).

3. EPIDEMIOLOGICAL STUDIES

3.1. Outbreaks of viral gastroenteritis

3.1.1. Two outbreaks of gastroenteritis caused by viruses typically associated with waterborne transmission have been described (Taylor *et al*, 1993; Wolfaardt *et al*, 1995). Although it has not been possible to prove any role of water in the transmission of the viruses concerned, the incidents show that outbreaks typically associated with water do occur in South Africa.

3.1.2. The annual January Dusi Canoe Marathon has been studied with regard to clinical and serological symptoms of canoeists and accompanying persons, and the quality of the river water (Grabow, 1993; Grabow *et al*, 1994; Taylor *et al*, 1995). The results suggested meaningful risks for some infections, but not for viruses such as hepatitis E and Norwalk.

3.1.3. In August 1994 some data were gathered on a typically waterborne outbreak of gastroenteritis in Knysna. Gastroenteritis viruses were detected in stool specimens of some patients. Enteric viruses were isolated from the drinking water supply. The water also contained levels of faecal coliforms, enterococci, and somatic and male-specific coliphages in excess of limits recommended for drinking water. Faecal pollution was ascribed to an error in the drinking water supply system (unpublished data).

3.2. General incidence of astroviruses

Astroviruses were detected in about 6% of more than 1000 stool specimens from gastroenteritis patients, predominantly children (Taylor *et al*, 1993; Marx *et al*, 1995). This indicates that the incidence of these viruses typically associated with waterborne transmission is similar to that in many other parts of the world, which implies that the risk of waterborne transmission must be similar to that in countries where waterborne transmission is on record.

3.3. General incidence of caliciviruses

Analysis of more than 1000 stool specimens from gastroenteritis patients, including hospitalised children and adults, some of which cases from two outbreaks, indicated that the incidence in South Africa of caliciviruses such as Norwalk virus, was similar to that in other parts of the world where these viruses are typically associated with waterborne transmission (Taylor *et al*, 1995; Wolfaardt *et al*, 1995).

3.4. Incidence of hepatitis E virus

A seroprevalence study on more than 800 healthy students and canoeists revealed that almost 3% of them had antibodies to the hepatitis E virus. This implies that they must have had an hepatitis E infection at some stage (Grabow *et al*, 1994). Incidences of hepatitis E antibodies of up to 14% have since been reported by other laboratories for certain communities in South Africa. This leaves no doubt that the virus must be present in the country. However, no clinical cases, other than a few imported cases, have yet been described in South Africa. This situation remains unexplained. However, the presence of the virus in the country implies that the possibility of waterborne outbreaks similar to those typical for other parts of the world cannot be ignored (Grabow *et al*, 1996, Appendix 6).

4. STUDIES ON DIFFUSE EFFLUENTS

4.1. Dusi Canoe Marathon

Seroprevalence studies failed to detect a meaningful association of hepatitis A and E, and Norwalk virus infections, with canoeing in the rivers concerned (Grabow *et al*, 1994; Taylor *et al*, 1995). the only meaningful serological association was for bilharziasis. However, data derived from questionnaires submitted by canoeists and accompanying persons indicated that canoeists had a significantly higher incidence of clinical symptoms typical of waterborne infections than accompanying persons with less exposure to the river water (unpublished data). At various sampling sites the levels of faecal pollution in the rivers exceeded limits generally recommended for recreational use (Grabow, 1993).

4.2. Botshabelo

A comprehensive study on the microbiological quality of diffuse effluents from informal settlement portions of Botshabelo and their impact on receiving water sources has been reported in detail (Jagals, 1994; Jagals *et al*, 1995). In principle the results revealed heavy faecal pollution, particularly in stormwater run-off, which has far-reaching implications for the safety of the receiving water sources. No enteric viruses were detected in a representative number of samples, even from water with heavy faecal pollution in terms of bacterial and phage indicators of faecal pollution. This may be due to a number of reasons which have not been investigated in any detail. The findings are important and further studies are envisaged. In this project reliable techniques have been established for distinction between faecal pollution of human and animal origin, which has valuable implications for assessment of health risks associated with faecal pollution of water supplies (Craun *et al*, 1994a; Grabow, 1996).

4.3. Mamelodi Stanza Bopape Village

This area was selected as a typical site for research on the impact of diffuse effluents on the microbiological quality of receiving waters. An excellent working relation and infrastructure has been established with the city councils of Pretoria and Mamelodi, relevant civic organisation, the

local community, and hospitals and clinics in the area. A detailed report is presented in Appendix 6. Heavy faecal pollution of receiving waters has been recorded. In contrast to diffuse effluents at Botshabelo, all diffuse effluents from Stanza Bopape Village contained high levels of cytopathogenic viruses in addition to bacterial and phage indicators of faecal pollution. This is the most comprehensive study on viruses in wastewater carried out to date in South Africa, and on diffuse effluents from informal settlements anywhere in the world. The results emphasise the health risk constituted by diffuse effluents from informal settlements, and underline the importance of appropriate sanitation for the communities concerned (Craun *et al*, 1994a; Grabow, 1996). Unfortunately it was not possible to obtain sufficient data on infections in patients for meaningful correlation with the quality of wastewater.

5. GENERAL DISCUSSION AND CONCLUSIONS

Valuable progress has been made in the development of techniques for the recovery of viruses. The glass wool adsorption-elution procedure for the economic, simple, in-line and on-site recovery of viruses from water has been successfully introduced for water quality monitoring. The affinity chromatography procedure for the selective recovery of viruses of choice from water is ready for application in studies on for instance epidemiological studies on waterborne outbreaks and research on specific viruses.

The molecular techniques which have been developed for the detection of viruses which are not detectable by conventional methods open doors for research on viruses which has not previously been possible in South Africa. Application of the techniques in practice has provided valuable details on the incidence and potential waterborne transmission of viruses such as calici, astro, rota, enteric adeno and hepatitis E.

The impact of diffuse effluents from informal settlements on receiving waters has been studied in detail at two typical model situations, namely Botshabelo and Mamelodi Stanza Bopape Village. Indirectly related information has also been obtained from the Dusi Canoe Marathon with recreational exposure to river water subject to faecal pollution from sources including diffuse effluents from informal settlements. The results show that these effluents carry heavy loads of faecal pollution, and in at least some cases, also heavy loads of viruses. These findings confirm health risks constituted by diffuse effluents from informal settlements and underline the importance of appropriate sanitation for the communities concerned.

An infrastructure and expertise has been established for further research on health aspects of waste waters, water sources and drinking water supplies. Various research needs and priorities have been identified. Among these are research on risks of infection and the safety of water supplies with regard to viruses which were not previously detectable. The project has made a valuable contribution to capacity building on health aspects of water which are of fundamental importance to the water industry and to the control of waterborne diseases in South Africa.

Further details are outlined in appendices and research outputs of publications, conference presentations and theses listed elsewhere in this report.

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Waterborne diseases: Update on water quality assessment and control

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Abstract

Water-borne diseases are the most important concern about the quality of water. The pathogens involved include a wide variety of viruses, bacteria and protozoan parasites. Due to differences in size, structure, composition and excretion by humans and animals, their incidence and behaviour in water environments differ. This constitutes difficult challenges for testing the safety of water and the efficiency of treatment processes. Further complications are that many water-borne pathogens, notably the great majority of viruses as well as protozoan cysts and oocysts, are not readily detectable. In addition, the prevalence of various water-borne pathogens changes as selective pressures change. In view of the diverse and variable goalposts, new epidemiological data, and progress in technology and expertise, the methods and strategies for quality monitoring and control of water-borne diseases are continually being revised and updated. This paper reviews the latest approaches to water quality monitoring using indicators of human and animal faecal pollution, and new methods for the detection of viruses. The importance of simple, economic and rapid methods for high frequency basic monitoring of water quality and the efficiency of treatment systems is emphasised. Reference is made to the fundamental need for microbiological quality data in the management of national and regional water resources and supplies.

Introduction

In a keynote address at the prestigious 1993 Stockholm Water Symposium, international authority Hillel Shuval illustrated the impact of water-borne diseases by comparing it to a 747 jumbo jet carrying 400 children and 100 adults crashing with no survivors every half hour around the clock (Editorial, 1993). This illustration is based on authentic estimates that some 50 000 people die each day in the world due to water-borne and water-related diseases (Ince, 1990; Schalekamp, 1990; Catley-Carlson, 1993). Extreme examples include the outbreak of 300 000 cases of hepatitis A and 25 000 cases of viral gastroenteritis in Shanghai caused in 1988 by shellfish harvested from a sewage-polluted estuary (Halliday et al., 1991). In 1991 an outbreak with 79 000 cases of hepatitis E in Kanpur was ascribed to polluted drinking water (Ray et al., 1991; Grabow et al., 1994), and in 1993 some 403 000 cases of cryptosporidium diarrhoea were caused by a conventional drinking water supply in Milwaukee, USA (MacKenzie et al., 1994). Although the mortality of many waterborne diseases is relatively low, the socio-economic impact even of non-fatal infections is phenomenal (Avendano et al., 1993; Payment, 1993). Further details on the public health and socio-economic implications of pathogenic micro-organisms in water, and the extent to which they outweigh the impact of diseases associated with the chemical quality of water, have been reviewed elsewhere (Bern and Glass, 1994; Craun et al., 1994a; b).

Little information is available on water-borne diseases in South Africa. This is probably due to the absence of an infrastructure for the detection and recording of such infections. The lack of information tends to create a false sense of security. There is no reason to believe that risks of water-borne diseases are any different from those in the rest of the world. In terms of escalating demands and pollution of the limited water sources, particularly in rural and developing communities, the risk may even be relatively high. This possibility is supported by data which show correlations of enteric infections in various communities to levels of sanitation, standard of living and education. The data reflect the incidence and public health impact of these diseases in the country (Von Schirnding et al., 1993). Anecdotal data and unpublished findings also point towards water-borne diseases.

The water industry has a long history of research and development aimed at supplying safe water and controlling water-borne diseases. In modern times certain principles for the treatment and disinfection of water have become established. However, evidence is mounting that drinking-water supplies which have been treated by processes generally accepted as sufficient and meeting conventional guidelines for bacterial indicators of faecal pollution, may play a meaningful role in the transmission of pathogens (Hejkal et al., 1982; Zmirou et al., 1987; Gerba and Haas, 1988; Bosch et al., 1991; Payment et al., 1991; Regli et al., 1991; MacKenzie et al., 1994). According to Payment et al. (1991) conventionally treated drinking water may be responsible for as much as 35% of household infectious gastroenteritis. The great majority of infections associated with drinking water which met criteria based on faecal bacteria, were caused by viruses and protozoan parasites (Grabow, 1991; Regli et al., 1991; Moore et al., 1994). These observations disclose shortcomings in quality surveillance programmes often used.

Since world-wide there would not seem to be a meaningful decline in the significance of water-borne diseases, research on fail-safe treatment technology and reliable quality monitoring continues (Grabow, 1986; 1990; Gerba and Haas, 1988; Regli et al., 1991; Bellamy et al., 1993; Sobsey et al., 1993; Craun et al., 1994b). The challenges to accomplish these goals increase in complexity as populations of humans and domestic animals increase with concomitant escalation in demand for potable water and pollution of limited water resources. Special efforts are required to control water-borne diseases in developing communities and countries, which are most vulnerable to these diseases (Feachem, 1980; Feachem et al., 1983; Catley-Carlson, 1993).

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This paper highlights progress in technology and approaches to quality monitoring and assessment of treatment efficiency, and outlines strategies to ensure the safety of water supplies.

Water purification and disinfection technology

A wide variety of water treatment systems and disinfection processes is available (Grabow, 1990; Bellamy et al., 1993; WHO, 1993; 1996; Schutte, 1995). At the low technology and inexpensive end of the range are methods such as the boiling or simple sand filtration of water, the addition of household bleach to a bucket of drinking water, storage of water, and the exposure of water to sunlight. At the sophisticated end of the range are multiple-barrier treatment trains capable of the direct reclamation of drinking water from waste water (Grabow, 1991; Regli et al., 1991).

Principally and theoretically all of these systems are capable of producing safe water supplies. However, without exception they are subject to breakdown and human failure in operation, supervision and quality monitoring (Grabow, 1990; Regli et al., 1991; Bellamy et al., 1993; Sobsey et al., 1993). Despite all efforts, there is no indication that we shall ever have treatment systems or disinfection processes which ultimately are not subject to failure of some kind or another. This implies that reliable quality monitoring of water sources and treated supplies will remain of fundamental importance in the control of water-borne diseases.

Water-borne and water-related pathogens

Water-borne diseases are typically caused by enteric pathogens which belong to the group of organisms basically transmitted by the faecal-oral route. In other words, they are mainly excreted in faeces by infected individuals, and ingested by others in the form of faecally contaminated water or food. Some of the pathogens may be of animal origin. Some may also be transmitted by personal contact, droplet transfer, or inhalation of contaminated aerosols. Water may also play a role in the transmission of pathogens which are not faecally excreted. These include opportunistic pathogens which are members of the normal flora of the external human body. Some of these pathogens are released into water from wounds, lesions or ulcers. Some opportunistic pathogens are natural inhabitants of certain water environments.

Assessment of the public health impact of water-borne diseases is complicated by factors such as:

- Many infections are not readily diagnosed, and detection of the aetiological agents in water is even more difficult. Water-borne transmission and assessment of the public health impact are, therefore, difficult to prove. The same applies to infections which have long incubation periods or manifest in long-term effects (Grabow, 1990; Craun, 1991; Craun et al., 1994a). For instance, viruses are estimated to play a role in about 20% of all cancer cases, and well-known oncogenic viruses such as papilloma, polyoma and hepatitis B are excreted in urine and other body fluids. However, epidemiological association of the diseases with water-borne transmission would be difficult (Grabow, 1990). Proving the water-borne transmission of pathogens is as difficult as proving the reverse, in other words, proving where people with infectious diseases got their infections from. Evaluation of available data and new information leave little doubt that the role of water in the transmission of pathogens and the public health impact of the infections are underestimated (Gerba and Haas, 1988; Payment et al., 1991; Moore et al., 1994; Gerba et al., 1995). These conclusions are supported by recent data on complications and manifestations of infections by coxsackie and other enteroviruses which are not readily associated with water-borne transmission (Clements et al., 1995; Gerba et al., 1995).
- Many water-borne infections, particularly viral infections in children, do not cause clinical disease. However, the pathogens are replicated and excreted by the infected individuals, which constitutes a risk of infection to others (Gerba and Haas, 1988).
- The impact of infectious water-borne diseases is aggravated by infected individuals who transmit the pathogens to other people by routes such as personal contact. Secondary and even tertiary transmission of water-borne diseases has been confirmed epidemiologically (Morens et al., 1979). Obviously, it is difficult to detect this indirect transmission of pathogens by water, and it becomes virtually impossible when the primary infection contracted from water was sub-clinical.
- The prevalence of different water-borne pathogens changes as selective pressures in various communities and parts of the world change (Hughes and La Montagne, 1994). For instance, in the USA the transmission of most bacterial pathogens by water decreased extensively in recent years due to more efficient treatment of water. However, the relative role of viruses, protozoan parasites and some bacteria such as *Escherichia coli* O157:H7 has increased due to factors like higher resistance to treatment processes (Craun, 1991; Moore et al., 1994; Craun et al., 1994a; Gerba et al., 1995). *Cryptosporidium parvum* was not generally accepted as a human pathogen until about 1976; it was associated with water-borne transmission for the first time in 1985; and in 1993 it caused the Milwaukee outbreak referred to earlier, which is the largest water-borne disease outbreak on record (Sobsey et al., 1993; MacKenzie et al., 1994).
- The incidence and prevalence of water-borne pathogens is subject to geographical factors. Most of the pathogens are distributed world-wide, but outbreaks of some, for instance cholera and hepatitis E, tend to be regional (Grabow et al., 1994). Dracunculiasis is restricted to rural areas in India and Pakistan, and Nigeria and certain other sub-Saharan countries (WHO, 1993). The complexity is illustrated by recent findings that the hepatitis E virus is endemic in South Africa, but clinical cases or outbreaks common in some parts of the world have so far been extremely rare in the country (Grabow et al., 1994; 1996).
- Individuals in various communities are not equally susceptible to water-borne infections. Persons with increased risk of infection as well as severity of disease include the very young and the elderly, pregnant women, the immunocompromised (e.g. organ transplant, cancer and AIDS patients), those predisposed to other illnesses (e.g. diabetes), and those with a chemical dependancy (e.g. alcoholism). In the USA these individuals at increased risk of water-borne diseases constitute almost 20% of the total population (Haas et al., 1993). This percentage is growing, and in some other countries, notably the developing world, the percentage may be considerably higher.

One implication of the above is that data on the incidence of water-borne disease, the importance of various pathogens potentially transmissible by water, and the risk of infection, cannot be extrapolated from one part of the world, country or community to another. The wide variety of pathogens which may be transmitted by water has been reviewed (Casemore, 1991; Sobsey et al., 1993; WHO, 1993; 1996; Grabow et al., 1994). The following is a summary of typical representatives and examples:

Enteric pathogens typically transmitted by the faecal-oral route

Bacteria

Salmonella spp., *Shigella* spp., pathogenic *Escherichia coli*, *Campylobacter* spp., *Vibrio cholerae* and *Yersinia enterocolitica*.

Viruses

Rotaviruses, enteric adenoviruses, caliciviruses (including "small round structured viruses" such as Norwalk, Snow Mountain, Hawaii, SA Christmas, SA Congress, etc.), astroviruses, "small round viruses", enteroviruses, coronaviruses, hepatitis A and E viruses, etc.

Protozoa

Entamoeba histolytica, *Giardia intestinalis*, *Cryptosporidium parvum*.

Opportunistic pathogens

Infections of the skin and mucous membranes of the eye, ear, nose and throat:

Pseudomonas aeruginosa, and species of *Mycobacterium*, *Aeromonas*, *Flavobacterium*, *Acinetobacter*, *Klebsiella* and *Serratia*.

Infections contracted by the inhalation of contaminated aerosols:

Legionella spp. (legionellosis), *Naegleria fowleri* (primary amoebic meningo-encephalitis) and *Acanthamoeba* spp. (amoebic meningitis, pulmonary infections).

Larval stages of parasites

Infections contracted by exposure to, or ingestion of, infectious larval stages of human parasites released by specific snails or water fleas:

Schistosoma spp. (schistosomiasis, bilharziasis) and *Dracunculus medinensis* (dracunculiasis).

The latter is not faecally excreted but typically transmitted by water and of major public health importance in restricted geographical areas (WHO, 1993).

Toxins from cyanobacteria

Toxins released by blooms of cyanobacteria (blue-green algae) such as *Microcystis aeruginosa* may adversely affect the health of animals and possibly also humans.

Nuisance organisms

A variety of non-pathogenic micro-organisms, and small plants and animals, may under undesirable conditions thrive in water supplies and cause turbidity, taste and odour, or visible animal life, which are aesthetically objectionable.

Indicator organisms

Commonly used indicators

The detection of many water-borne and water-related pathogens requires expensive and time-consuming techniques, while others are not detectable by conventional methods at all. It would, therefore, hardly be possible to include tests for all, or even a meaningful representation, of these pathogens in routine quality surveillance. Water quality monitoring programmes are, therefore, usually based on tests for indicator organisms. The primary objective of indicators commonly used is to indicate faecal pollution. The following are some of the most important requirements of faecal indicators (Grabow, 1986; 1990; WHO, 1993):

- Present whenever pathogens are present
- Present in the same or higher numbers than pathogens
- Specific for faecal or sewage pollution
- At least as resistant as pathogens to conditions in natural water environments, and water purification and disinfection processes
- Non-pathogenic
- Detectable by simple, rapid and inexpensive methods.

Ideally, various other properties are desirable, such as counts which are directly related to those of pathogens. However, in view of major differences in features such as structure, composition, size, resistance, and excretion by humans and animals, no such correlations exist. These differences also explain shortcomings of faecal bacteria as indicators for pathogens such as viruses and protozoan cysts or oocysts. At best commonly used indicators such as coliform bacteria can, therefore, be expected to indicate relatively recent faecal pollution, which reflects the potential presence or absence of enteric pathogens.

Many micro-organisms have attractive indicator features. However, they all have advantages and disadvantages, and there is no single indicator that universally meets all requirements. The reliability of indicators is evaluated by comparison of their incidence and survival in water environments and treatment processes to that of pathogens, as well as epidemiological studies on the consumers of water supplies, calculations based on the minimal infectious dose of pathogens, and experiments using human volunteers (Grabow, 1990; Payment et al., 1993; Sobsey et al., 1993; Graham et al., 1994).

The following is a summary of key features of commonly used indicators and new indicator concepts:

Total coliform bacteria

The term "total coliform bacteria", sometimes abbreviated to "total coliforms", "coliforms", "colis", etc., refers to a vaguely defined group of Gram-negative bacteria primarily identified by the ability to ferment lactose with the production of acid and gas, or aldehyde, within 24 h at 35 to 37°C. These organisms have a long history in water quality assessment, mainly because of their association with faecal pollution, and relatively easy and rapid detection. Some members of the group are almost conclusively of faecal origin, while others may also multiply in suitable water environments. Total coliforms are primarily used for assessment of the general sanitary quality of finally treated and disinfected drinking water (SABS, 1984; Grabow, 1990; WHO, 1993; 1996; *Standard Methods*, 1995).

The test method generally recommended is membrane filtration using M-Endo agar LES and incubation for 24 h at 35 to 37°C (Grabow and Du Preez, 1979; SABS, 1984; ISO, 1990; *Standard Methods*, 1995). Alternative agar media, pads saturated with liquid media, or most probable number (MPN) tube dilution procedures which yield comparable results, are acceptable for certain purposes. Confirmation (verification, differentiation) of coliform bacteria by various procedures including an oxidase test, production of acid and gas from lactose, and possession of the enzyme ß-D-galactosidase, is often recommended (ISO, 1990; *Standard Methods*, 1995). One reason for confirmation is to exclude cytochrome

oxidase-positive bacteria (mainly *Aeromonas* species) which produce coliform-like colonies on Endo and certain other media. Exclusion of cytochrome-oxidase positive bacteria is preferred primarily because most of these bacteria generally detected are members of the natural flora of water environments and not of faecal origin. Exclusion of these organisms is, however, debatable for the following reasons:

- *Aeromonas* and related bacteria are opportunistic pathogens known to cause gastroenteritis and wound infections, which implies that their presence in drinking water constitutes a potential health risk (Grabow and Du Preez, 1979; Burke et al., 1984; Moyer, 1987).
- *Aeromonas* bacteria are excreted by infected individuals which implies that their presence may indicate faecal pollution.
- Many oxidase-negative coliforms may also multiply in water environments, e.g. *Klebsiella* species (Grabow, 1990; WHO, 1993; *Standard Methods*, 1995).
- Properly treated drinking water is free of *Aeromonas* and related bacteria, which implies that their presence indicates treatment or disinfection failure, or aftergrowth in distribution networks (Grabow and Du Preez, 1979; Seidler et al., 1981; Burke et al., 1984; Grabow, 1990).
- Confirmatory procedures increase the time (additional 2 to 3 d), cost and labour of coliform tests, which implies that the attractive feature of a relatively simple, inexpensive and rapid test is lost.

In view of the above considerations it would seem advisable to use membrane filtration and incubation on M-Endo agar LES for 24 h at 35 to 37°C as a practical test for routine quality monitoring. Any colonies with the typical golden-green metallic sheen should be taken as indication of unacceptable quality and alert for more detailed investigation. This could include picking the colonies from the membrane for further testing to establish potential faecal origin, and immediate testing of another sample from the same source, including additional tests for faecal pollution. The sensitivity of analysis could be increased by filtering larger volumes of water. In the basic coliform test 100 mℓ and even 500 mℓ samples which fail to pass through 0.45 µm pore-size membranes, fail to comply with drinking water quality guidelines for turbidity or suspended matter. Overgrowth which interferes with typical coliform colonies indicates unacceptable quality due to shortcomings in treatment and disinfection, or aftergrowth (Grabow, 1990). This approach to coliform testing is substantiated by research and views of others, including Seidler et al. (1981), Burke et al. (1984), SABS (1984), Moyer (1987) and States and Sykora (1995).

Additional procedures for coliform tests are sometimes recommended, such as tests for "injured" coliforms, pre-incubation at lower temperatures, delayed coliform tests, rapid tests, etc., which have advantages for certain purposes (*Standard Methods*, 1995). Unfortunately, however, all the different media and test procedures tend to confuse the non-expert. Another important disadvantage is that the population of organisms recorded as "coliforms" by the different tests is not necessarily the same. The extent to which counts and populations differ for various water environments is not clear, but it may be meaningful (Grabow and Du Preez, 1979; Grabow, 1990; ISO, 1990; *Standard Methods*, 1995). This variation implies that coliform counts recorded by different methods may not be directly comparable. In addition, there is no meaningful evidence that any of these modifications make a significant contribution to the basic total coliform test recommended above (*Standard Methods*, 1995). In case of doubt about the sensitivity of the basic coliform test for chlorinated drinking-water supplies, the volume of test water could simply be increased to 500 mℓ or even 1 000 mℓ or more which would compensate for the modifications while the test remains simple, economic and rapid (Grabow, 1990).

The recently introduced chromogenic substrate coliform test has attractive features for routine quality monitoring (*Standard Methods*, 1995; States and Sykora, 1995). The test consists of adding test water (100 mℓ or other volume) to a suitably selective growth medium which contains the hydrolysable chromogenic enzyme substrates ortho-nitrophenyl-ß-D-galactopyranoside (ONPG) and 4-methyl-umbelliferyl-ß-D-glucuronide (MUG). During incubation for 24 to 28 h at 35 to 37°C the enzyme ß-D-galactosidase specific for total coliforms hydrolyses ONPG with release of the yellow chromogen orthonitrophenol. At the same time the enzyme ß-glucuronidase specific for *Escherichia coli* hydrolyses MUG, releasing the fluorogen which fluoresces brightly under an ultraviolet light lamp (wavelength 365 nm). The enzymes are highly specific and no confirmation is required. Oxidase-negative bacteria such as *Aeromonas* and *Pseudomonas* fail to produce sufficient quantities of the enzyme ß-D-galactosidase to yield a yellow colour under the specified test conditions. The one test, therefore, simultaneously indicates the presence of total coliforms (yellow colour) and *E. coli* (fluorescence). The test is relatively simple and economic, yields results within 24 to 28 h, and can be carried out as a quantitative MPN tube dilution procedure or a qualitative presence-absence test, both of which have attractive features for routine quality monitoring. Suitable growth media are commercially available under trade names such as "Colilert". Despite attractive features, the method has shortcomings. In at least one study a substantial proportion of *E. coli* isolates (mean 34%, median 15%) from human faecal samples were found to be negative for the ß-D-glucuronidase enzyme (Chang et al., 1989). This implies that results may underestimate counts of *E. coli* and differ from results obtained by other methods.

Faecal coliform bacteria

This term refers to certain members of the group of total coliform bacteria which are more closely related to faecal or sewage pollution, and which generally do not readily multiply in water environments. This group of bacteria is also known as thermotolerant coliforms or presumptive *E. coli*, and outdated terminology includes faecal E. coli, faecal coli, etc. Faecal coliforms are primarily used for the assessment of faecal pollution in waste water and raw-water sources. They are detectable by simple and inexpensive tests, and are widely used in routine water quality monitoring (Grabow et al., 1981; SABS, 1984; Grabow, 1990; ISO, 1990; 1994a; *Standard Methods*, 1995). The generally recommended test method is membrane filtration with M-FC agar (or alternative media which yield equivalent results) and incubation for 24 h at 44.0 ± 0.5°C. This incubation temperature is usually referred to as 44.5°C. The membrane filtration procedure has the advantage that individual colonies can be identified, and the presence of *E. coli* is almost conclusive evidence of faecal pollution.

Escherichia coli

This species is a member of the group of faecal coliform bacteria. Outdated terminology includes "*E. coli* type I" and "confirmed *E. coli*". *Escherichia coli* has the important feature of being highly specific for the faeces of humans and warm-blooded animals. For

all practical purposes these bacteria fail to multiply in any natural water environment, and they are, therefore, used as specific indicators for faecal pollution (SABS, 1984; Grabow, 1990; ISO, 1990; 1995a; WHO, 1993; 1996; *Standard Methods*, 1995). *Escherichia coli* is traditionally detected by carrying out a test for faecal coliforms, followed by testing isolates for the ability to produce indole from tryptophan within 24 h at 44.5°C. The latest trend is towards methods in which *E. coli* is identified directly by detection of the enzyme ß-glucuronidase, similar to the chromogenic substrate test described earlier for total coliforms. A neat system in which dilutions of test water (200 µℓ) are inoculated into wells of 96-well microtitre plates containing dehydrated growth medium and 4-methyl-umbelliferyl-ß-D-glucuronide (MUG) is under consideration (ISO, 1994a). The plates are incubated for 36 h at 44.5°C, positive wells identified in a UV observation chamber (ultraviolet light at 366 nm) and calculation of the MPN by computer program. Since 8 wells are used per dilution, the MPN value is relatively accurate.

Enterococci

Enterococci, also referred to as faecal streptococci, are related groups of bacteria which are more closely associated with faecal pollution than total coliform bacteria because members typically present in faeces of humans and animals do not readily multiply in water environments. Recently the term "faecal enterococci" (previously "intestinal enterococci") has been proposed for a group consisting exclusively of *Enterococcus faecalis*, *E. faecium*, *E. durans* and *E. hirae*, which are highly specific for human and animal faecal pollution (ISO, 1994b). These spherical Gram-positive bacteria tend to be more resistant than faecal coliforms which are Gram-negative. Enterococci are detectable by practical techniques, such as membrane filtration using selective media like M-Enterococcus agar and incubation for 48 h at 35 to 37°C (Grabow, 1990; *Standard Methods*, 1995). An attractive new method for faecal (intestinal) enterococci, similar to the chromogenic substrate MPN procedure using 96-well microtitre plates described earlier for *E. coli*, is under consideration (ISO, 1994b). Detection is based on the ability of the organisms to hydrolyse 4-methyl-umbelliferyl-ß-D-glucoside (MUD) with release of the fluorogen in the presence of thallium acetate and nalidixic acid within 36 h at 44.5°C.

Heterotrophic plate count

This test, which was previously known as the total or standard plate count, detects a wide variety of organisms, primarily bacteria, including organisms of faecal origin, as well as natural inhabitants of water environments. It is primarily used to assess the general microbiological quality of finally treated and disinfected drinking water supplies. The test is simple and inexpensive, yields results in a relatively short time, and has proved one of the most reliable and sensitive indicators of treatment or disinfection failure (Grabow et al., 1980; Grabow, 1986; 1990; WHO, 1993; 1995; *Standard Methods*, 1995). One reason is that highly resistant bacterial spores are also detected. The generally recommended test method is pour plates using a rich growth medium such as Yeast Extract Agar and incubation for 48 h at 35 to 37°C.

Clostridia

An important advantage of these Gram-positive anaerobic bacteria is that their spores are more resistant to conditions in water environments, as well as treatment and disinfection processes, than most pathogens, including viruses (Grabow, 1990; Payment and Franco, 1993; WHO, 1993; 1996). Clostridia are sometimes considered as too resistant, and their inclusion in water quality guidelines as too stringent. One of the members of the group, *Clostridium perfringens* is, like *E. coli*, highly specific for faecal pollution. According to Payment and Franco (1993) *C. perfringens* is the most reliable indicator for viruses and protozoan cysts or oocysts in treated drinking water. Clostridia generally occur in lower numbers in waste water than coliform bacteria, and detection methods are relatively expensive and time-consuming (Grabow, 1990; Payment and Franco, 1993; WHO, 1993; 1996).

Acid-fast bacteria

This term refers to extremely resistant members of the group of mycobacteria, including *Mycobacterium fortuitum* and *M. phlei*. The bacteria proved most useful for assessment of the efficiency of treatment trains for the direct reclamation of drinking water from waste water (Grabow, 1990).

Other indicators

A variety of other indicators has been used in water quality assessment, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Legionella* species, *Candida albicans*, and endotoxins. All of these have advantages for certain purposes (Grabow, 1986; 1990; *Standard Methods*, 1995).

Distinction between faecal pollution of human and animal origin

Batteries of indicator organisms have been selected for the distinction between faecal pollution of human and animal origin. The distinction is based on *Rhodococcus coprophilus* which is specific for animal faecal pollution, sorbitol-fermenting bifidobacteria and phages of *Bacteroides fragilis* HSP40 which are specific for human faecal pollution, and the ratio of faecal coliforms to enterococci which may also give an indication of the origin of faecal pollution (Jagals et al., 1995). Identification of the origin of faecal pollution may be important for various purposes. For instance, the risk of human infection is generally higher with human than with animal faecal pollution (Jagals et al., 1995).

Protozoan parasites

The cysts and oocysts of intestinal parasites such as *Giardia* and *Cryptosporidium* species are exceptionally resistant (Casemore, 1991; Jakubowski et al., 1991; Bellamy et al., 1993). They generally occur in low numbers in water environments, and are not readily detectable. However, their minimal infectious dose may be as low as a single cyst or oocyst. Since it is difficult to detect the low numbers in water which may constitute a health risk, quality control is often based on specifications for raw water quality and the efficiency of treatment processes rather than testing of the treated water (Regli et al., 1991; Bellamy et al., 1993).

Human viruses

Many viruses may potentially be transmitted by water. Most of them are exceptionally resistant to treatment processes and their minimal infectious dose may be as low as a single particle. It is, therefore, not surprising that they cause the majority of water-

borne infections (Haas et al., 1993). Their relative importance tends to increase due to water treatment and disinfection practices which remove or inactivate more sensitive pathogens and bacterial indicators of faecal pollution but not viruses (Craun, 1991; Regli et al., 1991; Sobsey et al., 1993). Another important feature of viruses is that their incidence in water environments may differ substantially from that of indicators such as coliform bacteria for reasons such as:

- Viruses are excreted only by infected individuals and coliform bacteria by virtually all humans and warm-blooded animals. Numbers of viruses in water environments are, therefore, generally lower than those of indicators such as faecal coliforms by several orders of magnitude.
- Viruses are excreted for relatively short periods in numbers of up to 10^{12}/g of faeces, while coliform bacteria are excreted fairly consistently in numbers of about 10^{8}/g of faeces.
- The structure, composition, morphology and size of viruses differ fundamentally from those of bacteria, which implies that behaviour and survival in water differ extensively.

Due to the above differences bacteria such as coliforms have shortcomings when being used as indicators for viruses. Ideally water quality surveillance should, therefore, include tests for viruses. Unfortunately, however, tests for viruses are relatively expensive, complicated and time-consuming, and require sophisticated facilities and expertise. In addition, the great majority of viruses concerned are not detectable by conventional virological cell culture techniques (Taylor et al., 1993; Grabow et al., 1994; Wolfaardt et al., 1995). Control of the virological safety of water is, therefore, as in the case of intestinal parasites, often based on raw water quality and specifications for purification and disinfection processes rather than testing of the treated water (Regli et al., 1991; Sobsey et al., 1993).

New developments in quality surveillance

Bacteriophages

Bacteriophages (phages) are viruses which infect bacteria. In terms of size, structure, morphology and composition they closely resemble human viruses. The behaviour of phages in water and related environments, and their resistance to unfavourable conditions, treatment systems and disinfection processes do, therefore, more closely resemble those of human viruses than bacterial indicators of faecal pollution (Grabow et al., 1984; 1993; Havelaar et al., 1993; ISO, 1995b; 1995c).

Phages only replicate in specific host bacteria, which implies that the phages of *E. coli* (coliphages) are, like their hosts, related to faecal pollution. Phages commonly used in water quality assessment include the groups of phages known as somatic and male-specific coliphages, which each have their own indicator advantages and disadvantages (Grabow et al., 1980; 1984; 1993; Havelaar et al., 1993). Phages which infect *Bacteroides fragilis* strain HSP40 are highly specific for human faeces, and can be used to distinguish between faecal pollution of human and animal origin (Tartera and Jofre, 1987; Grabow et al., 1995).

Virological analysis of water

Challenges in the virological monitoring of water quality include the recovery of small numbers of viruses from large volumes of water, the detection of a wide variety of viruses, and reduction of the cost and time of testing. The following progress has recently been accomplished:

Recovery of viruses

A filter system for the practical and economic on-site and in-line recovery of viruses from water supplies has been developed and evaluated (Grabow and Taylor, 1993). The perspex filters contain oiled sodocalcic glass wool with electrostatic and hydrophobic properties suitable for the efficient adsorption of viruses at pH levels of up to 9.0 (Vilagines et al., 1993). Dechlorination granules for the neutralisation of chlorine residuals in the water under investigation are placed over the glass wool. The filters can easily be attached to water supplies by means of conventional quick-fit domestic garden hose fittings. Any desirable volume of water can on site be passed through the filters. The filters (dimensions 26 cm x 3 cm, mass 100 g) are then transported in cooler bags to the laboratory where viruses are eluted. Efficiency of recovery is at least 50%, which is better than that of much more expensive adsorption-elution systems generally used.

Detection of viruses

Viruses in water are generally detected by propagation in cell culture systems such as the BGM monkey kidney cell line (Regli et al., 1991). According to the latest evidence the detection of these cytopathogenic viruses can be increased considerably by using the BGM cell line, primary vervet kidney cells and the PLC/PRF/5 human liver cell line in parallel with three blind sub-passages (Grabow et al., 1996). However, many water-borne viruses are not cytopathogenic in presently available cell culture systems and are, therefore, not detectable by conventional cell culture propagation. Progress is now being made in the development of techniques for detecting many non-cytopathogenic viruses. These techniques are mainly based on molecular methods for the highly specific detection of viral nucleic acid. One of these techniques is the polymerase chain reaction (PCR), which can be used for the direct detection of viruses (Marx et al., 1995; Wolfaardt et al., 1995), or detection of some viruses after cell culture amplification (Grabow et al., unpublished). The latter approach increases the sensitivity of detection, and overcomes the disadvantage of molecular techniques of not being able to distinguish between viable and non-viable viruses.

The above developments imply that relatively practical, inexpensive and sensitive procedures are now available for the recovery of viruses from water, and for their detection by cell culture isolation and molecular techniques. In the past the running cost of virus tests by cell culture isolation exceeded that of coliform tests by the order of 10 to 100 (Grabow, 1986). This has now been reduced to the order of 5 to 10. In addition, virological water analysis has become much more sensitive and user friendly (Grabow and Taylor, 1993).

Water quality surveillance

Monitoring the safety of water supplies

Despite modern technology for efficient treatment and disinfection, the transmission of diseases by water does not only continue, but in at least some situations seems to increase or become more complicated due to selection for resistant or "new" pathogens (Grabow, 1989; Casemore, 1991; Craun, 1991; Regli et al., 1991; Hughes and La Montagne, 1994). Breakdown in treatment plants,

and human error in operation and supervision, generally take place without warning; in fact, like a thief in the night they tend to strike when least expected. Routine quality monitoring should, therefore, be carried out at the highest possible frequency in order to detect problems at the earliest possible stage. Since monitoring programmes are subject to many variables, including economic considerations, it is not possible to formulate universal sampling frequencies, and each case has to be considered on its own merit. Generally speaking it is better to run simple and inexpensive tests at high frequency, than long, complicated and expensive tests at low frequency (Haas, 1993; WHO, 1993; 1996).

No single indicator can fulfil all the needs of water quality monitoring. Each indicator has its own advantages and disadvantages. Best results are, therefore, obtained by using combinations of indicators for different purposes (Grabow, 1990). For instance, indicators selected for routine monitoring of treated drinking water supplies may include a basic 24-h test for total coliform bacteria, supplemented by tests for faecal coliforms or E. coli, and possibly somatic coliphages and the heterotrophic plate count (Grabow, 1990). These tests, supported by regular sanitary surveys and inspection of treatment procedures, would detect errors and potential risks in reasonable time, and make a major contribution to the control of water-borne diseases (Grabow, 1990; WHO, 1993; 1996). Practical methods for routine quality monitoring are particularly important for laboratories with limited financial resources, facilities and expertise. This typically includes laboratories of small communities and developing countries, which are particularly vulnerable to water-borne diseases (Feachem, 1980; Feachem et al., 1983; Catley-Carlson, 1993; WHO, 1993).

Increasing evidence of the risks of water-borne diseases and their public health impact, as well as shortcomings of commonly used indicators, justify supplementary tests on the quality of raw and treated waters, and the efficiency of treatment systems, wherever possible. It would seem logical for the water industry and health authorities to calculate expenditure on microbiological water quality monitoring as a percentage of the total budget for water supply, duly taking into account the potential price of disease and consumer perception of efforts to ensure the safety of water supplies.

Microbiological data for management of water resources and supplies

As a result of increasing demands and concomitant pollution of the environment, the regional and national management of water resources and supplies has become essential. This applies in particular to a country such as South Africa with limited and variable natural water resources, and an escalating population coupled with rapid growth in industry and agriculture. Details on microbiological quality constitute an integral part of data required for management systems. This information gives an indication of the suitability of water sources and supplies for various purposes, as well as treatment required to render water suitable for human and animal consumption. The utilisation of a conceptual framework on water quality is strongly recommended because traditional practice based on observing contamination and water-borne outbreaks of disease, and applying engineering technology for control, is clearly inadequate because the transmission of pathogens by water does not only continue, but would seem to increase in many areas (Sobsey et al., 1993).

Details on the quality of water sources and the treatment required have financial implications for the supply of safe water. These details are also essential with regard to pathogens such as viruses and protozoa which are difficult to detect in treated water, and quality assessment largely depends on information on the raw water quality and the efficiency of the treatment processes applied (Regli et al., 1991; Bellamy et al., 1993). The identification of pollution sources is required for the protection of water resources, and for the necessary pollution control. Details on the quality of water sources and supplies have major benefits for research on the epidemiology of water-borne diseases and infection risks associated with the utilisation of water for various purposes, including drinking, recreation and irrigation. The information is also essential for research on the survival of pathogens and indicators in water environments, the efficiency of treatment processes, and the development of reliable methods for water quality monitoring (Regli et al., 1991; Bellamy et al., 1993; Sobsey et al., 1993; Moore et al., 1994).

Technical details on the collection and processing of data for management systems are not discussed here. However, in terms of methods for microbiological water quality monitoring discussed earlier, it is important to note that the need for data based on practical, economic and comparable analyses has been emphasised (Sobsey et al., 1993; States and Sykora, 1995).

Management systems based on a conceptual framework of microbiological water quality and modelling of health risks are, therefore, essential for the optimum utilisation of available water resources, the protection of these resources, the economic supply of safe water, and the control of water-borne diseases. These considerations have far-reaching implications. For instance, access to safe water is considered a human right (ANC, 1994). The need for such management systems is widely recognised, and details on approaches and applications have been described elsewhere (Lloyd and Bartram, 1991; Regli et al., 1991; Leahy et al., 1993; Sobsey et al., 1993; Craun et al., 1994b; Moore et al., 1994; States and Sykora, 1995).

Water quality guidelines and standards

Water quality guidelines and standards recommended by various authorities and countries differ. This is primarily due to perceptions of acceptable risks in terms of economic considerations and technical capabilities, and the quality and quantity of available raw water sources (Feachem, 1980; Shuval et al., 1981; Feachem et al., 1983; Regli et al., 1991; Sobsey et al., 1993; WHO, 1993; 1996).

Authorities are cautious in defining acceptable risks of infection, but the following proposals serve as valuable guidelines:

- Maximum acceptable risk for drinking-water supplies recommended in the USA (Regli et al., 1991):
  One illness per 10 000 consumers per year.
- Maximum acceptable risk for the recreational use of environmental waters (not swimming pools) recommended in the USA (Environmental Protection Agency, 1986) and Canada (Canadian Guidelines, 1987):
  One illness per 1 000 swimmers.

Based on considerations such as the above, guidelines and standards for water have been formulated by many authorities, and are continually being revised and updated as new information and technology become available (SABS, 1984; Regli et al., 1991; Water Affairs Guidelines, 1993; WHO, 1993; 1996; *Standard Methods*, 1995). Although these guidelines and standards differ in technical details, they generally share certain basic requirements. For instance, they all specify that drinking water should rarely if ever contain total coliform bacteria, and never faecal coliforms or *E. coli*. In addition, guidelines and standards generally specify or

assume that drinking water should be free of pathogens such as viruses and protozoa (European Communities, 1980; Helmer et al., 1991; WHO, 1993; 1996; *Standard Methods*, 1995). However, limits are rarely specified for viruses and protozoa because testing remains complicated and expensive, and beyond the capabilities of most laboratories involved in conventional routine water quality monitoring. Efforts to increase the reliability of monitoring for viruses and protozoa, include the application of additional indicators such as phages (European Communities, 1980; Grabow et al., 1993; *Standard Methods*, 1995) and clostridia (European Communities, 1980; Payment and Franco, 1993), as well as specifications for raw water quality and the efficiency of treatment processes (Regli et al., 1991). However, as progress is being made in the development of more practical techniques, additional analyses are recommended. For instance, tests for viruses have been included in specifications for recreational waters (European Communities, 1975), and examination of drinking water supplies for pathogens including viruses and protozoan parasites has been recommended (European Communities, 1980; Smeets and Amavis, 1981; Regli et al., 1991).

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Appendix 2

New methods for the recovery and detection of viruses

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NEW METHODS FOR THE VIROLOGICAL ANALYSIS OF DRINKING WATER SUPPLIES

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KEY WORDS
Drinking water, viral infections, viruses, water quality, indicators, bacteriophages, recovery, glass wool, adsorption-elution, detection, cell cultures.

INTRODUCTION

Drinking water supplies have a long history of involvement in the transmission of infectious diseases. Traditionally waterborne diseases are associated with contaminated or untreated supplies. However, evidence is accumulating that even drinking water supplies treated by methods generally accepted as sufficient may play a meaningful role in the transmission of pathogens (Hejkal *et al*, 1982; Akin, 1984; Bitton *et al*, 1986; Rose *et al*, 1986; Bosch *et al*, 1991a; Craun, 1991; Payment *et al*, 1991; Regli *et al*, 1991). The data show that viruses are of major importance in this regard (Williams and Akin, 1986; Grabow, 1991), and confirm shortcomings in the reliability of indicators such as coliform bacteria widely used for monitoring the safety of drinking water supplies. Increasing attention is, therefore, world-wide being given to the virological analysis of drinking water, particularly supplies reused indirectly or directly (Grabow, 1990; Payment *et al*, 1991; Regli et al, 1991; American Public Health Association, 1992).

Challenges in the virological monitoring of water quality include the recovery of small numbers of viruses from large volumes of water. This is because the minimal infectious dose of the viruses concerned may be as low as a single virus particle, and numbers of viruses are generally lower than those of indicators such as faecal coliforms by several orders of magnitude (Regli *et al*, 1991). The second major challenge is the detection of viruses. The great majority of the viruses concerned are not detectable by conventional methods such as propagation in cell cultures. Both the recovery of viruses and their detection require advanced technology, know-how and facilities which imply that virological monitoring tends to be expensive.

This paper describes progress in technology development with regard to practical and economic techniques for the recovery of viruses from water supplies, sensitive detection methods, and the detection of a wide range of viruses. Implications are that virological testing is now within closer reach of routine programmes for water quality monitoring.

METHODS FOR VIROLOGICAL MONITORING

Recovery of viruses

A filter system for the practical and economic on-site and in-line recovery of viruses from water supplies has been developed and evaluated. The perspex filters contain oiled sodocalcic glass wool with electrostatic and hydrophobic properties suitable for the efficient adsorption of viruses at pH levels of up to 9,0. On top of the glass wool, the filters contain dechlorination granules for the neutralisation of chlorine residuals in the water under investigation. The filters can easily be attached to water supplies by means conventional quick-fit domestic 13mm garden hose fittings. A flow meter can be used to record the volume of water that flows through the filter over a desired period of time which may be as long as 24h under suitable conditions. The filters (dimensions 26cm x 3cm, mass 100g) are then transported in cooler bags to the laboratory where viruses are eluted by means of a pH 9,5 glycine-beef-extract buffer. This filter system, which eliminates the transportation of large volumes of water to the laboratory, is a modification of principles developed and extensively tested by Vilagines *et al* (1988, 1993). Efficiency of recovery is at least 50%, which is considerably more than that of other adsorption-elution systems used for routine monitoring in a number of countries (American Public Health Association, 1992).

Detection of viruses

The BGM monkey kidney cell line is generally used for the detection of viruses recovered from water (American Public Health Association, 1992). Primary vervet kidney cells are more sensitive but not readily available because they are derived directly from monkey kidneys (Grabow and Nupen, 1981). A search for alternative cell culture systems has revealed that the PLC/PRF/5 cell line derived from a primary human hepatocellular carcinoma would appear to be more sensitive than BGM cells, and probably even primary vervet kidney cells (Grabow *et al*, 1983, 1992). The PLC/PRF/5 cell line has other advantages too, including considerably lower cost than primary vervet kidney cells. In comparative studies on polluted rivers in Transvaal and Natal, 32 viruses were isolated, 22 of which on PLC/PRF/5 cells and 10 on BGM cells, which reflects the superiority of the former for the detection of naturally occurring viruses (Table 1). The great majority of isolates have so far been typed as members of the group of enteroviruses; further typing of these isolates is in progress.

Preliminary results of research aimed at further increasing the sensitivity of cell culture detection systems, indicate that at least in laboratory experiments the susceptibility of certain cell cultures to certain viruses may be increased up to 100-fold by treatment with agents such as 5-iodo-2-deoxy-uridine (Benton and Hurst, 1986). Research on other enhancing agents such as cholesterol, polyethylene imine and indomethacin (Khatib *et al*, 1990) is in progress. In these studies the modification of the suceptibility of the BGM, MA-104 and PLC/PRF/5 cell lines, as well as primary vervet kidney cells, to viruses including polio, coxsackie A and B, echo, reo, simian rota SA11, human rota HRV-3, enteric adeno and hepatitis A.

Progress has also been made in the development of techniques for the detection of viruses which fail to multiply in any cell culture systems presently available (Taylor *et al*, 1993). These techniques include immune electron microscopy, immunosorbent assays,

and molecular techniques. The latter include procedures known as gene probe hybridisation and the polymerase chain reaction (PCR) in which viruses are detected by highly specific identification of their nucleic acid. The occurrence of viruses in communities is also detectable by surveys of the presence of specific antibodies in members of the communities. Application of these techniques and approaches have made it possible for the first time in South Africa to directly detect potentially waterborne viruses such as Norwalk and astro (Taylor *et al*, 1993), and to obtain evidence for the presence of the hepatitis E virus. The latter virus causes hepatitis (jaundice) epidemiologically and clinically similar to that of the well known hepatitis A virus, and has caused waterborne outbreaks in certain parts of the world (Craske, 1992). The incidence in water environments of other viruses not detectable by conventional methods such as the human immunodeficiency virus, can also be investigated by means of currently available molecular techniques (Ansari *et al*, 1992).

CONCLUSIONS

Relatively practical, inexpensive and sensitive techniques are now available for the recovery of viruses from water, and for their detection by cell culture isolation. Substantial progress has also been made in the development of techniques for the detection of potentially waterborne viruses which has not previously been possible.

ACKNOWLEDGEMENTS

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Table 1. Human viruses recovered from rivers in Transvaal and Natal

Date	Isolate	River	Cell culture	Virus
92-10-27	UK1	Umlazi/Kwandengezi	PLC/PRF/5	Entero
	UK2	Umlazi/Kwandengezi	PLC/PRF/5	Entero
	UK3	Umlazi/Kwandengezi	PLC/PRF/5	Coxsackie B
92-10-29	UI1	Umlazi/Isipingo	PLC/PRF/5	Entero
	UI2	Umlazi/Isipingo	PLC/PRF/5	Entero
	UI3	Umlazi/Isipingo	PLC/PRF/5	Entero
	UI4	Umlazi/Isipingo	BGM	Coxsackie B
92-12-09	SL1	Slangspruit/Imbali	BGM	Reo
	SL2	Slangspruit/Imbali	BGM	Reo
92-10-12	KL1	Klip	PLC/PRF/5	Adeno
	KL2	Klip	PLC/PRF/5	Adeno
92-10-19	KL3	Klip	PLC/PRF/5	Entero
	KL4	Klip	PLC/PRF/5	Entero
	KL5	Klip	PLC/PRF/5	Entero
	KL6	Klip	BGM	Entero
	KL7	Klip	PLC/PRF/5	Entero
	KL8	Klip	PLC/PRF/5	Entero
92-11-09	KL9	Klip	PLC/PRF/5	Entero
	KL10	Klip	PLC/PRF/5	Entero
92-11-30	KR1	Klein Riet	PLC/PRF/5	Entero
	KR2	Klein Riet	PLC/PRF/5	Entero
	KR3	Klein Riet	PLC/PRF/5	Entero
	KR4	Klein Riet	BGM	Entero
	KR5	Klein Riet	PLC/PRF/5	Entero
	KR6	Klein Riet	BGM	Entero
	KR7	Klein Riet	BGM	Entero
92-12-07	KR8	Klein Riet	PLC/PRF/5	Entero
	KR9	Klein Riet	PLC/PRF/5	Entero
	KR10	Klein Riet	PLC/PRF/5	Entero
	KR11	Klein Riet	BGM	Entero
	KR12	Klein Riet	BGM	Entero
	KR13	Klein Riet	BGM	Entero

Appendix 3

Recovery of viruses by affinity chromatography
Modification of cell culture sensitivity to viruses
Selection of most sensitive cell culture system
Detection of enteroviruses by PCR and gene probe techniques
Genetic characterization of South African strains of hepatits A virus

Vth International Congress on the
Impact of Viral Diseases in the Developing World

Johannesburg, South Africa, 9-14 July 1995

Oral Paper

AFFINITY CHROMATOGRAPHY RECOVERY OF COXSACKIEVIRUS B1 FROM WATER USING POLI-OR MONOCLONAL ANTIBODIES. **N Potgieter*, WOK Grabow, JA Verschoor[1].** Department of Medical Virology and [1]Department of Biochemistry, University of Pretoria, Pretoria, South Africa

Immuno-affinity chromatography (AC) refers to the specific recovery of viruses of choice by passing test suspensions through columns in which antibodies (Abs) directed against the viruses are immobilised on a suitable matrix. The viruses are eventually released by appropriate buffers. Available data are largely limited to the qualitative recovery of viruses using polyclonal Abs (PAbs). The recovery of viable viruses has not yet been reported. We have compared the quantitative recovery of viable and non-viable coxsackievirus B1 from water by means of PAbs and monoclonal antibodies (MAbs) coupled to CNBr-activated sepharose 4B, and MAbs coupled by disulphide linkages to sepharose 4B, using selected buffers for elution. In all systems more than 99% of viable viruses in seeded 200 ml samples of tapwater were retained by the antibodies. However, commonly used elution buffers inactivated the viruses. Best results were obtained with pH 4,5 citrate buffer, which recovered viable viruses at an average efficiency of 0,4%. Optical density readings at 260 nm on eluates indicated that substantial quantities of viral proteins were recovered. Eluates from CNBr-coupled MAbs contained 59% more viral proteins than eluates from conventional systems of CNBr-coupled PAbs, and 54% more than eluates from disulphide-coupled MAbs. Recovery of intact viral particles was confirmed by electron microscopy.

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Oral Paper

RESEARCH ON THE MODIFICATION OF CELL CULTURE SENSITIVITY FOR THE DETECTION OF ENTERIC VIRUSES. **KL Botma*, WOK Grabow.** Department of Medical Virology, University of Pretoria, Pretoria, South Africa

Certain compounds have been reported to enhance the susceptibility of cell cultures to certain viruses. We have investigated the value of cell culture modification for the isolation of viruses from environmental samples. In tests on laboratory strains of viruses, treatment of the BGM and FRhK4-R monkey kidney cell lines with 5-iodo-2'-deoxyuridine (IDU), increased cytopathgenic effect (CPE) titres of polio vaccine strains 1 and 3, echo-1, coxsackie A9 and B1, and hepatitis A virus strain pHM-175, by 10- to 100-fold. IDU also increased titres of simian rotavirus SA11 on the MA104 monkey kidney cell line by up to 10-fold. In addition, treated cells displayed an earlier and more pronounced CPE. Cholesterol or indomethacin increased titres by 5- to 10-fold. Polyethylene-imine (PEI) had no meaningful effect. IDU-treatment of the PLC/PRF/5 human liver carcinoma cell line slightly reduced titres of adenovirus types 40 and 41. In studies on naturally occurring viruses in sewage, treatment of cells had no meaningful effect on sensitivity or titres. However, CPE was more pronounced, and on average detectable 2 days earlier, on IDU-treated monkey kidney and PLC/PRF/5 cell lines. Unfortunately, however, IDU-treatment also increased the sensitivity of cells to toxic effects. The results indicate that IDU-treatment has certain benefits for the cell culture detection of enteric viruses in environmental samples.

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Poster Presentation

COMPARISON OF SEVEN CELL CULTURE TYPES FOR THE ISOLATION OF ENTERIC VIRUSES.

N Potgieter*, WOK Grabow. Department of Medical Virology, University of Pretoria, Pretoria, South Africa

A wide variety of cell culture types is being used for the isolation of cytopathogenic enteric viruses. The sensitivity of different cell types for various viruses is known to differ considerably. In an attempt to find the most sensitive system for the isolation of viruses from environmental samples which may contain low numbers of viruses or viruses with impaired infectivity, we have compared seven cell culture types most likely to meet this requirement. Comparative titrations using confluent cell monolayers in microtitre plates and calculation of the most probable number titre by standard computer programme, were used in tests on water samples seeded with laboratory strains of viruses, and samples of sewage or polluted river water. The Buffalo Green Monkey Kidney (BGM) cell line, the PLC/PRF/5 human liver cell line and primary vervet kidney (PVK) cells proved more sensitive to laboratory strains of poliovirus 1 (vaccine strain), coxsackievirus A9 and B1, echovirus 1 and reovirus 1 than primary human embryonic fibroblasts, the MA104 and FRhK-4R foetal rhesus kidney cell lines, and the Caco-2 colon carcinoma cell line. In comparative tests on samples of domestic wastewater and polluted river water from various sources, viruses were detected more often and titres were higher on the former three cell types than the rest. There was no consistent correlation of viruses recovered on cell types, and best results were obtained by using BGM, PLC/PRF/5 and PVK cells in parallel.

Oral Paper
Berg-en-Dal Virology Congress
22-25 August 1993

THE DETECTION OF ENTEROVIRUSES BY MEANS OF PCR AND GENE PROBE TECHNIQUES

M WOLFAARDT and W O K GRABOW

Department of Medical Virology, University of Pretoria

Recent progress in the development of molecular techniques has facilitated approaches to new techniques with attractive features for the detection of enteroviruses. The purpose of this study was to investigate the application of the polymerase chain reaction (PCR), and hybridization with gene probes, for the detection of enteroviruses in faecal specimens and environmental samples, including wastewater and shellfish meat.

Oligonucleotide primers, based on published nucleotide sequences, were selected from the highly conserved 5′ nontranslated region of the enterovirus genome to detect the widest possible range of enteroviruses by PCR amplification with a product size of 194 base pairs. A vaccine strain of poliovirus type 1 was used as the model to optimise detection methods and to assess the sensitivity of the methods. Tenfold dilutions of cell culture stocks of the poliovirus, titrated by cytopathogenic end-point, were seeded into preparations of human faeces, sewage, and homogenates of shellfish meat. Each sample was tested by PCR and cell culture propagation. RNA was extracted from samples by conventional phenol/chloroform procedures, and incubated for 30 min at 56°C in the presence of CTAB to remove inhibitors of cDNA synthesis. Conventional techniques were used for the production of cDNA. Forty cycles of PCR amplification were carried out using procedures optimised for the test samples concerned. The PCR products generated with the enteroviral primers were analyzed by agarose gel electrophoresis and the specificity of the amplification confirmed by hybridization with an internal gene probe. Poliovirus was successfully detected in all test samples. Similar results were obtained in comparative tests on samples seeded with coxsackie A9 and coxsackie B1 to B6 viruses, and echovirus types 1 to 3. Hepatitis A virus was used as a negative control. PCR detection proved at least as sensitive as cell culture propagation for enteroviruses which readily produce a cytopathogenic effect in monkey kidney cells.

The results indicate that PCR can successfully be used for screening a wide variety of samples for the presence of enteroviruses. Research is now in progress to ascertain the spectrum of enteroviruses detectable by this method, including enteroviruses which do not readily multiply in cell cultures. This relatively fast and sensitive detection method, together with type-specific primers and probes, may eventually be used for diagnostic purposes, and to screen environmental samples for enteroviruses.

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Oral Paper

GENETIC CHARACTERIZATION OF SOUTH AFRICAN STRAINS OF HEPATITIS A VIRUS

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Hepatitis A virus (HAV) is associated with endemic and epidemic hepatitis A worldwide, and common source outbreaks can often be attributed to faecally contaminated food and water. Human isolates are of a single serotype, with four genotypes within the one serotype. There are an additional three genotypes associated with Old World monkeys. Investigations have shown that in some geographic regions related strains are clustered suggesting endemic spread of the virus, while in other regions several genotypes are circulating. Genetic analysis of strains from both patient and environmental specimens can therefore provide valuable information with regard to the source of the virus. As far as we are aware no HAV strains from South Africa have been typed to date. The aim of this investigation was to characterize HAV strains from South African clinical specimens. Using published primers designed to amplify the VP1 region, HAV RNA was amplified by reverse transcriptase polymerase chain reaction. The amplified PCR products were sequenced directly by chain termination sequencing, and the sequences compared with published HAV sequences to determine their genetic relatedness. Sequences from clinical isolates from two outbreaks showed ≥90% and ≥88% homology with published sequences for HAV strains HM-175 and MBB respectively. This suggests that the South African isolates sequenced to date cluster within the same genotype, ie genotype I, as strains MBB and HM-175.

Appendix 4

Rapid characterization of viruses by immunoelectron microscopy

THE RAPID CHARACTERISATION OF COXSACKIE B VIRUS AND POLIOVIRUS ISOLATES FROM WATER SAMPLES BY IMMUNOELECTRON MICROSCOPY

M B Taylor and L Engelbrecht (unpublished)

The virus family *Picornaviridae* is possibly the largest of all the virus families. Within this family is the *Enterovirus* genus which contains many human pathogens. These viruses are transmitted predominantly by the faecal-oral route. As the name implies, the enteroviruses inhabit the enteric tract and are remarkedly resistant to acid pH's, proteolytic enzymes and bile salts. Within this genus are 69 viruses known to infect man and historically these viruses were allocated to three groups - polioviruses, coxsackieviruses and echoviruses. The distinction between the coxsackieviruses and echoviruses is not absolute, consequently newly discovered enteroviruses have just been allocated an enterovirus number beginning at enterovirus 68. Within each historical group are a number of serotypes, i.e. Poliovirus 1-3, Coxsackievirus A1 - A24 (no A23) & B1 - 6, Echoviruses 1 - 34 (no 10 or 28) and the new enteroviruses 68 - 71 (72 was assigned to the hepatitis A virus). In general, coxsackieviruses are more highly transmissable than echoviruses as they appear to be shed for longer in the faeces and respiratory secretions.

Most enteroviruses, excluding some of the coxsackie A viruses, are readily isolated in cell culture, but the cytopathic effects and inclusion bodies are identical for all isolates. Coxsackieviruses differ from the other enteroviruses in that they are pathogenic for newborn mice, and coxsackie A viruses can be differentiated from the coxsackie B viruses by the type of paralysis caused in the mice. The differentiation between serotypes of enteroviruses is dependant on time consuming neutralisation tests using expensive polyvalent pools of horse sera especially prepared for this purpose.

Immunoelectron microscopy (IEM) is a rapid reliable technique which can be used to serotype viruses from both clinical and environmental specimens. It has the advantage that it can be applied to unpurified virus preparations, of unknown virus titre, such as found in cell culture fluid. In most cases, a positive result is visualized as virus-antibody aggregates, wheras a negative result is indicated by

predominantly single virus particles. The results are also available within 3 - 4 hours compared with the 4 - 8 days required for the neutralisation assay. Caution must however be exercised in interpreting IEM results as many viruses tend to clump naturally in the absence of antibody. In these cases however no "halo" of antibody can be visualized around the virus particles.

The agar-diffusion IEM technique described by Cubitt *et al* (1979) was used to identify the Coxsackie B and polioviruses among 49 enteroviral isolates from rivers in Transvaal and Natal. Reference pooled Coxsackie B virus and poliovirus were diluted as with other typing methods, and the dilution (1:10) which gave the optimum IEM results using cell culture fluid from laboratory strains of Coxsackie B 1 - 6 and vaccine strains of poliovirus 1-3 was used for all further studies. Positive and negative controls were run concurrently with the isolates from the water samples.

Ten of the 49 isolates were positively identified as Coxsackie B virus while 2 isolates appeared to be polioviruses. The observed aggregates of virus visualised in the latter two specimens must however have been due to natural aggregation, as confirmatory poliovirus neutralisation assays were negative for poliovirus.

Reference:

Cubitt WD, McSwiggan DA, Moore W (1979): Winter vomiting disease caused by calicivirus. Journal of Clinical Pathology 32:786-793.

Appendix 5

Detection of hepatitis E virus by the polymerase chain reaction

Detection of the hepatitis E virus by the polymerase chain reaction

F E Marx and W O K Grabow

Department of Medical Virology, University of Pretoria
December 1994
(unpublished report)

INTRODUCTION

Hepatitis E or enterically transmitted non-A, non-B hepatitis has long been known as an important cause of morbidity and mortality in man (Favorov *et al.,* 1994). It is caused by an RNA virus transmitted via the faecal-oral route (Bradley, 1992). It usually occurs as an outbreak of waterborne hepatitis in certain developing countries with poor sanitation, and is endemic in some of these regions (Nanda *et al.,* 1994; Tucker and Kirsch, 1994).

PHYSICAL PROPERTIES

Hepatitis E viruses (HEV) are spherical, 27 -34nm, non-enveloped single stranded RNA viruses (Bradley, 1992; Tsai *et al.,* 1994; Tucker and Kirsch, 1994). The HEV particles have spikes and indentations on the surface. The virus is sensitive to high salt concentrations, proteolytic digestion and freeze-thawing. HEV has a 7.5kb, single stranded, positive-sense RNA genome with three overlapping open reading frames (ORFs) (Balayan, 1993; Favarov *et al.,* 1994). The non-structural genes are located at the 5′ end of the genome and the structural genes at the 3′ end. Most of the structural proteins are coded within ORF2, but all three frames contribute to the morphology of HEV (Balayan, 1993; Bradley 1992).

Earlier studies suggested that HEV should be classified as a member of the picornavirus group, but morphology and other properties resemble those of the caliciviruses. Analysis of the non-structural genes, however, have indicated similarity to the α-like virus supergroup which includes rubella (Tucker and Kirsch, 1994). At this stage HEV is, therefore, classified as a member of the Caliciviridae.

EPIDEMIOLOGY AND MODE OF TRANSMISSION

HEV has been responsible for several massive outbreaks of hepatitis in certain areas of the world (Bi et al., 1993). Many of these outbreaks have occurred as a result of sewage leaking into river water or failure of the chlorination process for drinking water (Tucker and Kirsch, 1994). HEV can be seen as the principal agent for endemic hepatitis in developing countries as outbreaks have been reported from Mexico, India, China, Nepal, Bangladesh, Pakistan, Indonesia etc. In Africa outbreaks have occurred in Algeria, Ghana, Ethiopia, Somalia and the Ivory Coast (Balayan, 1993; Bradley, 1992; Grabow *et al.,* 1994; Ramalingaswami and Purcell, 1988).

Groups of people in temporary settlements, e.g. refugee camps and military personnel, as well as staff members treating patients with acute HEV are at increased risk of contracting HEV (Tucker and Kirsch, 1994). Sporadic hepatitis due to HEV infection has been described in India (Ray *et al.,* 1994). No epidemics of acute HEV have yet been documented in southern Africa, but the first indication of HEV was a typical waterborne outbreak in Botswana in 1988 (Byskov *et al.,* 1989). Based on epidemiology, clinical symptoms, and elimination of other potential causes HEV could be implicated as the probable cause. The first known case of HEV infection in South Africa was a pregnant woman in Cape Town who probably contracted the virus during a visit to Bombay, India in 1992 (Robson *et al.,* 1992).

A hepatitis E seroprevalence study undertaken by the Department of Medical Virology, University of Pretoria, indicated for the first time that HEV might be endemic in South Africa, as 2.05% from a total of 782 individuals, tested positive for anti-HEV antibodies (Grabow *et al.,* 1994). Preliminary data from a recent study undertaken in the Cape Province support this finding as 5.8 - 14.3 % of black South Africans in that region have antibodies to HEV (Tucker and Kirsch, 1994).

South Africa has various areas where sanitation is poorly developed or non-existent, and furthermore there are informal settlements with inadequate sanitary facilities. These facts together with the findings that hepatitis E may be endemic in South Africa make a potential outbreak a definite possibility. With this in mind, precautions are a necessity and this includes means of fast and rapid detection of HEV.

Since the virus is not detectable by propagation in cell culture or infection of conventional laboratory animals (Nanda *et al.,* 1994), a sensitive HEV detection method is necessary. We have decided to develop a polymerase chain reaction (PCR) for the detection of HEV in samples from patients and the environment as it is a rapid and sensitive method that has previously been used with success (Bi *et al.,* 1993; Jothikumar *et al.,* 1993; Nanda *et al.,* 1994; Ray *et al.,* 1991).

MATERIALS AND METHODS

SAMPLES

Stool samples containing HEV were kindly donated by colleagues in the USA and Germany. The first stool sample was from an outbreak in India and was donated by Dr M O Favorov of the Centres for Disease Control in Atlanta, USA. Amplification of the HEV DNA through the reverse transcriptase polymerase chain reaction (RT-PCR) was attempted. The RT-PCR and the primers used were based on an article by Jothikumar *et al.* (1993). The oligonucleotide primers were synthesised by the Department of Biochemistry, University of Cape Town. RT-PCR amplification of the viral genome previously proved to be difficult with clinical samples (Bi *et al.,* 1993), and we also experienced problems with this method. Despite modifications to the technique and various unsuccessful attempts, amplification of the DNA could not be achieved.

In July 1994 we visited the Department of Medical Virology at the University of Tübingen in Germany, where Dr C-H Wang asssited us in mastering skills for the PCR detection of HEV. We successfully isolated HEV RNA from a stool sample and amplified a selected region of the genome. We used the primers EF1 and EF2 described by Sheng-Li *et al.* (1993). The experiments were subsequently successfully repeated in our own laboratory.

Dr S Emerson (USA) kindly donated a cDNA clone in a plasmid that contains cDNA from a large region of the genome of the Pakistani strain Sar-55. The cDNA is from a non-structural region in the open reading frame ORF1 from approximately nucleotide 3200 to 4950. The plasmid sequence of the HEV differs from the published PCR consensus sequence at various positions. The plasmid vector is pCR1000 and was grown in *E. coli* SC-1 cells under Kanamycin selection.

RNA EXTRACTION

Total RNA was extracted from 300μl of the stool samples using the method of Jiang *et al.* (1992) with one modification, ie back-extracting the freon phase with 200μl distilled water. The resultant supernatant was added to the supernatant from the first freon extraction. AV1-specific cDNA was prepared by reverse transcription (RT) and amplified by nested PCR using a modified technique based on the method described by Sheng-Li *et al.* (1993).

PRIMERS

The primer pair that we used was different from the pair used in Germany, but the sequence was obtained from the same article by Sheng-Li *et al.* (1993). This set of primers was specifically chosen to include a region of the cDNA clone. This enabled us to use the clone as a positive control. The primer pair amplifies a 1110bp region of the HEV genome. This region is situated in the ORF1, from nucleotide 3351 to 4461. The sequence of the primers are:
Forward primer (EF6): 5'-GGTTCTCCGTTGGTTGTTCTG-3'
Reverse primer (ER6): 5'-AGCACACTCTAGACCCAGAGAAAA-3'
The primers were synthesised by The Midland Certified Reagent Company of Midland, Texas, USA.

PCR REAGENTS AND CONDITIONS

Unless otherwise specified all PCR reagents used were from Boehringer Mannheim. Five microlitres of the test sample were used in a 100μl reaction volume consisting of the following RT-mixture (final concentration): 10x PCR buffer; 0.5mM dNTPs; 0.5μM primer HER6; 40U RNase inhibitor and 25U AMV reverse transcriptase. The reaction mixture was incubated at 42°C for 1 h. To the 100μl RT reaction mixture 0.5 μM primer HEF6 and 3.5U Taq DNA polymerase were added. The PCR reactions were carried out in a Hybaid thermocycler (Omnigene) with the following conditions: denaturation at 95°C for 4 minutes followed by 30 cycles of denaturation at 95°C for 30s, annealing at 51°C for 30s, elongation at 72°C for 40s and final extension at 72°C for 10 min. The resulting 1110bp PCR products were visualized by UV illumination of an 8% polyacrylamide gel stained with ethidium bromide. A positive control (cDNA clone) and negative control (distilled water) were included in each reaction. The PCR products of the stool samples co-migrated with the 1 110bp product from the positive control. Marker III was included in each gel to confirm the product size.

RESULTS

The A55 USA stool sample yielded a clearly defined 1110bp PCR product with the EF6 and ER6 primers. This product co-migrated with the cDNA positive control on a polyacrylamide gel (Fig 1). The stool sample from Germany, however, did not yield a positive band with these primers.

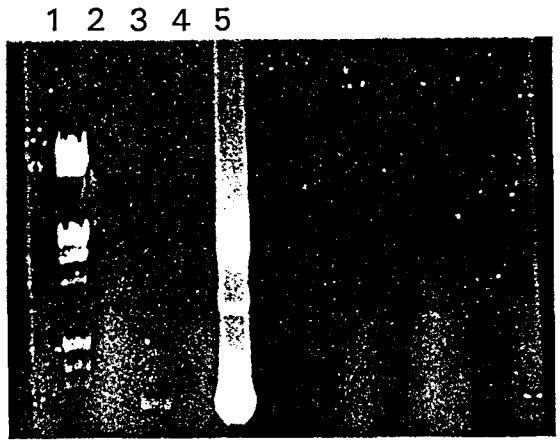

Figure 1: UV-illuminated gel of environmental samples tested with PCR.
Lane: 1 - Molecular weight marker III (Boehringer Mannheim); 2 - Negative control (distilled water); 3 - A 55 Indian stool (Favorov USA); 4 - German stool (Wang Germany); 5 - Positive control (cDNA USA)

We were able to extract RNA from stool samples and amplify the resulting DNA with the use of primers EF6 and ER6 in a modified RT-PCR reaction. The cDNA clone from Dr Emerson was successfully amplified and used as positive control. The A 55 Indian stool sample obtained from Dr Favorov yielded a clearly defined 1110bp PCR product that co-migrated with the positive cDNA control on a polyacrylamide gel (Fig 1). The stool sample obtained from Dr Wang in Germany failed to yield a positive band with this primer pair, but in Germany with the use of the EF1 and ER1 primer pair, a positive PCR product was obtained.

DISCUSSION

With the RT-PCR method described here we are now able to do a fast and accurate determination of the presence of HEV in stool samples. The fact that a positive PCR product could not be detected in the German stool sample with the use of the EF6 and ER6 primers, may be attributed to degradation of the virus during transport. It is known that the virus is sensitive to high salt concentrations, proteolytic digestion and freeze thawing (Tucker and Kirsch, 1994). A further possibility is that the EF1/ER1 primer pair used in Germany, may be more effective for the German sample. With the use of both primer pairs we should, however, be able to detect HEV in a variety of samples.

Future research will focus on improving the sensitivity of the method in order to determine the presence of HEV in environmental samples. We are also in the process of developing a RT-PCR reaction to determine the presence of HEV in seeded human liver samples.

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Appendix 6

Hepatitis E virus in South Africa

Hepatitis E virus in South Africa

W.O.K. Grabow, M.B. Taylor and L.M. Webber

The hepatitis E virus (HEV) and hepatitis A virus (HAV) are phylogenetically unrelated, but both cause typical outbreaks of clinical hepatitis associated with contaminated water and food. However, HAV causes outbreaks worldwide, but HEV only in certain areas, notably India and neighbouring countries, central Asia, China, Mexico and northern parts of Africa. Hepatitis E has major health implications in these countries and outbreaks with many thousands of cases are on record. Clinical cases are rare in parts of the world such as Europe, the UK, USA, South Africa, Australasia, Japan and South America. Seroprevalence studies using new technology now indicate that HEV may have a much wider distribution than previously thought. Recent surveys on populations in various parts of South Africa show sub-clinical infections in 2–15% of individuals. Reasons for the geographic association of clinical hepatitis E and outbreaks are not clear. Answers to these and related questions are essential for the formulation of strategies aimed at preventing the disease in countries such as South Africa, where cases are rare but conditions in many communities appear ideally suited for clinical disease and outbreaks.

Hepatitis E (enterically transmitted or epidemic non-A, non-B hepatitis) shares certain clinical and epidemiological characteristics with hepatitis A.[1,2] Both infections are primarily transmitted by the faecal-oral route, and often associated with waterborne outbreaks.[1,3] Hepatitis E tends to occur more often in young adults, however, many of whom are already immune to hepatitis A, with a mortality rate of up to 20% in pregnant women, a slightly longer incubation period (average about 40 days), and a lower secondary attack rate. Recent findings also suggest that, unlike other hepatitis viruses, the hepatitis E virus commonly causes intrauterine infection as well as substantial perinatal morbidity and mortality.[2] Although phylogenetically unrelated, the hepatitis E virus (HEV) and hepatitis A virus (HAV) are both typical non-enveloped single-strand RNA viruses with an icosahedral capsid of about 27–34 nm diameter.[4]

Hepatitis E infections and outbreaks have been reported from certain parts of the world, notably India, Nepal, Burma, Pakistan, Afghanistan, Borneo, parts of central Asia, China, Mexico, and African countries such as Egypt, Algeria, Ethiopia, Somalia, Sudan and the Ivory Coast.[3,5] The disease is endemic in many of these countries, and the most common cause of acute hepatitis in adults in parts of India, Asia and Africa.[2,5] Outbreaks associated with sewage-contaminated drinking water include one in 1955 involving an estimated 30 000 cases in Delhi, one in 1986–88 in the Xinjiang Uighar region of China with more than 100 000 cases, and one in 1991 with some 79 000 cases in Kanpur, India.[2,3] Apart from occasional imported cases, hepatitis E is not known to be endemic in parts of the world such as central Europe, Britain, North and South America, Australasia, Japan and South Africa.[1-3]

The first indication of hepatitis E in southern Africa was a typical waterborne outbreak with 273 cases and at least four deaths in 1985 in Maun, northern Botswana. Diagnosis was based on epidemiology, clinical symptoms, and elimination of other possible causes.[6] A similar outbreak with an unknown number of cases has also been reported for Namibia in 1983.[7] When diagnostic tests for anti-HEV recently became available, sera from both outbreaks were re-tested and HEV aetiology was confirmed by positive anti-HEV results.[7] The first known imported case of hepatitis E into South Africa was a 34-year-old pregnant woman who was admitted to the Groote Schuur Hospital in Cape Town in 1992 shortly after returning from holiday in Bombay, India.[8] The infection was secondarily transmitted to a doctor and two nurses, all of whom developed clinical hepatitis. We have recently diagnosed hepatitis E in a 45-year-old female admitted to the H.F. Verwoerd Hospital with clinical hepatitis shortly after returning from vacation in India (unpublished).

Information on the HEV and its epidemiology is limited because the virus and its antibodies are not readily detectable by conventional techniques.[4] However, detection methods based on molecular technology are now becoming freely available and rapid progress in research on the virus and the disease it causes is being accomplished worldwide.[1-3] This article deals with anti-HEV seroprevalence studies in South Africa, and an interpretation of the results regarding the risk of hepatitis E in resident communities.

Seroprevalence studies in South Africa

One study was carried out in 1992 on participants in the Dusi canoe marathon and on university students.[3] The 555 canoeists were predominantly healthy, strong males 20–40 years of age, selected at random to represent individuals with extensive exposure to river water polluted by sewage from low socio-economic communities potentially at high risk for HEV infection. This annual three-day white water canoe marathon is contested by about 1000 individuals in the Umsindusi and Umgeni rivers in Kwazulu-Natal.[9] In recent years a high incidence of infections typical of exposure to sewage-polluted water has been reported among participants.[9] For purposes of comparison, 227 healthy undergraduate medical students in our Faculty were included to represent individuals with minimal exposure to faecally polluted water. Most of the students were 19–22 years of age. All 782 serum specimens were screened for anti-HEV by means of a synthetic peptide enzyme immunoassay (EIA). Positive results were confirmed by a neutralization test using a mixture of five broadly immunoreactive HEV peptides. A Western blot (WB) procedure using HEV recombinant proteins as antigens was used for further confirmation as well as distinction between IgG and IgM antibodies.[3]

Ten of the 555 canoeists (1.8%), and 6 of the 227 students (2.6%) were anti-HEV positive. IgM anti-HEV antibodies were detected in 3 of the 6 students (50%) and in 1 of the 10 canoeists (10%). Demographic data on the positive individuals indicate local exposure to HEV. None of the 16 anti-HEV positive individuals had a record of clinical viral hepatitis which could be related to HEV infection. This is particularly significant for the 4 individuals who had IgM HEV antibodies, which implies that the infection must have been less than about 8 months prior to the collection of blood specimens.[1,3] It would appear, therefore, that all of them were sporadic cases of sub-clinical infection, the source of which is not evident. None of the 16 anti-HEV positive individuals was positive for any hepatitis B virus (HBV) or hepatitis C virus (HCV) markers, but 8 of them (50%) were anti-HAV positive,

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which is typical for comparable communities in the country.[3]

The observation that 2.05% of randomly selected individuals from two different communities were anti-HEV positive under circumstances which indicate local infection, suggests that HEV is endemic in South Africa. The results indicate that the virus occurs at relatively low levels as manifested by the occurrence of sporadic sub-clinical infections. The study population can be considered as fairly representative of middle to high socio-economic communities in the country. These communities are not considered to be at high risk for a disease which is primarily associated with inadequate public sanitation and malnourishment.[1,2,4,5]

Preliminary data of a second study on an unknown number of black adults over the age of 18 years in the Cape Province indicate an anti-HEV seroprevalence of 5.8% to 14.3%, with a higher incidence in rural areas.[10] None of the positive cases was associated with clinical disease or outbreaks, or with exposure to infection beyond the country's borders. These findings also suggest that the virus is endemic and spreads by sporadic sub-clinical infection.

A retrospective analysis of 131 stored serum samples from non-A, non-B, non-C (NANBNC) acute hepatitis patients admitted to Ga-Rankuwa Hospital north-west of Pretoria during 1991–92, using a commercial EIA for total HEV antibodies (Abbott Laboratories), yielded positive results for 44.3% of the cases.[11] All cases were members of local communities, with no history of travel abroad or contact with individuals who may have contracted HEV infection in other parts of the world. Using a developmental assay (Abbott), anti-HEV IgM was detected in 15.3% of the cases. These findings indicate that clinical hepatitis in the patients concerned was indeed hepatitis E. This would be the first evidence on record of clinical hepatitis E in the country due to locally contracted infection. None of the cases was associated with hepatitis outbreaks and the source of infection is unknown, which suggests that they were sporadic cases.

During the period June 1994 to July 1995, a total of 1143 sera submitted to our routine diagnostic laboratory were tested for anti-HEV using a commercial EIA for total HEV antibodies (Abbott Laboratories). The majority of the cases were from the H.F. Verwoerd and Kalafong hospitals. About 800 of the cases were from relatively low, and the rest from relatively high, socio-economic communities. Only 290 of the cases had primary liver pathology of some kind or other, while the rest had conditions with little if any relevance to hepatic infection. Nine (0.8%) of the cases yielded definitive positive results. Another 20 yielded borderline positive results which are under further investigation. One of the positive cases was a 52-year-old black female with jaundice, which could be a case of clinical hepatitis E. This study included the 45-year-old Indian female earlier referred to as a suspect imported case of hepatitis E. Clinical details on the remaining 7 anti-HEV positive cases do not indicate meaningful relation to hepatitis E infection. There was no indication of either association with an outbreak of viral hepatitis or source of infection for any of the anti-HEV positive cases.

Discussion

Results of seroprevalence studies presently available leave little doubt that HEV is endemic in South Africa. Some of the data suggest a higher incidence in lower socio-economic communities,[7,10,11] which would be typical of HEV epidemiology in parts of the world where the virus is endemic.[1–5] However, the data on canoeists and medical students suggest that infection also occurs in high socio-economic communities, including individuals with little if any exposure to sewage-polluted water or food.[3] Observations that the majority of anti-HEV positive cases in South Africa were 20 years or older[3,7–11] would also be in agreement with HEV epidemiology in some endemic parts of the world.[5] The anti-HEV seroprevalence in South Africa, which varied from less than 1% in certain communities to more than 14% in others, resembles that reported for other countries with low-level sub-clinical HEV infections.[2] This is substantially lower than in countries with endemic clinical HEV and epidemics. For instance, anti-HEV prevalence of up to 94% has been reported for villages in Somalia.[5]

Evidence which now indicates that HEV has a much wider distribution than previously thought, on the basis of clinical cases and outbreaks, raises important questions about the epidemiology of the virus. For instance, reasons for the high incidence of clinical hepatitis E with typical waterborne outbreaks in some areas, and predominantly sporadic sub-clinical infections in other parts of the world, remain unclear. Since the mode of transmission would seem to be basically similar for hepatitis E and A, it is difficult to understand why HEV is the most common cause of clinical viral hepatitis in some parts of the world, and rare in areas such as South Africa where HAV is endemic and clinical cases of hepatitis A common.[3] The difference may be due to strains of HEV with different levels of virulence which in some way or other are restricted to certain geographical areas. This would seem to be in agreement with imported cases of clinical disease in countries where clinical disease is rare.[2,8] Strain variation in HEV has been described — namely, the Mexico (M) and Burma (B) strains, and an atypical strain from north India which failed to react with typical anti-HEV serum.[1] Alternatively, the phenomenon may be due to predisposing conditions or factors in some parts of the world which promote clinical disease. Similar epidemiology is known for other viruses such as Epstein-Barr, and sporadic clinical cases restricted to certain geographical areas would also seem to be typical of the hepatitis F virus (HFV).[12] Rare incidence of clinical cases in countries such as South Africa may also be due to low prevalence of the virus, but it seems unlikely that diagnostic expertise would fail to detect even low numbers of clinical cases.

Concern about the virus is also based on recent findings that viraemia is common in patients and may last for up to 16 weeks, and that patients may excrete the virus in faeces for more than 7 weeks, well after clinical and biochemical recovery.[1,2] The period of viraemia has also been found to coincide with that of IgM and IgG response to the infection, and there are indications that acute phase immunoglobulin may lack neutralizing activity. It would also seem that some persons fail to produce an antibody response to HEV infection.[1] These features may be related to the ability of the virus to cause extensive outbreaks under suitable conditions, and may have implications for serological diagnosis of infection. Very little is known about the incidence and survival of the virus in water and food, which play a fundamental role in the transmission of the virus. Using the polymerase chain reaction, the virus has recently been detected in sewage in an endemic area of India, and some indications have been obtained on the removal of the virus by sewage treatment.[13] However, this is hardly a beginning to meaningful information required for controlling transmission of the virus by water and food.

Progress in research on the epidemiology of hepatitis E is awaited worldwide, but particularly in countries such as South

Africa where the virus would seem to be endemic, and conditions in many communities appear ideally suited for outbreaks of the disease. Concern is fuelled by an unconfirmed report that a hepatitis E outbreak involving more than 600 cases recently occurred in Namibia,[14] in the same area where a similar outbreak happened some 10 years ago.[7] Better understanding of reasons for the apparent geographic restriction of outbreaks is essentail for strategies aimed at the prevention of the disease in South Africa, which is the only method of control, since no vaccines or therapy are available.

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Appendix 7

Enteric viruses in diffuse effluents

Proceedings of the Biennial Conference of the Water Institute of Southern Africa, Port Elizabeth, South Africa, 20-23 May 1996. Water Institute of Southern Africa, Johannesburg (in press)

VIRUSES IN WASTE WATER FROM AN INFORMAL SETTLEMENT

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SUMMARY

Viruses are a major cause of waterborne diseases. Details on viruses in waste water are essential for management strategies aimed at the protection of water sources and the control of waterborne diseases. Virological screening of waste water is also carried out for monitoring enteric viruses circulating in communities, and assessment of public health campaigns such as poliomyelitis vaccination. In this study information has been obtained on viruses in diffuse effluents from a typical low socio-economic informal settlement with restricted sanitation and water supply. Sewage from an adjacent community with a sewerage system has likewise been analysed. The study area was selected as representative of communities which tend to be exceptionally vulnerable to enteric infectious diseases, and waste water from the settlements may be expected to contain high levels of waterborne pathogens. A total of 209 samples collected over a period of 9 months in 1994 were analysed for cytopathogenic enteric viruses and related indicators. Conventional cell culture propagation using three cell types yielded 486 isolates of viruses which consisted of 263 coxsackie B viruses (54%), 109 polioviruses (22%), 101 untyped enteroviruses (21%), 9 adenoviruses (2%), 2 echoviruses (<1%), and 2 reoviruses (<1%). All six types of coxsackieviruses were isolated, but 48% were type B2. All three types of polioviruses were isolated, all of them vaccine strains. Counts of faecal indicators and the incidence of viruses tended to increase after rainfall, which indicates that stormwater run-off was heavily polluted. Even though viruses were not enumerated, the isolation of viruses from 72 % of stream samples and 96% of sewage samples, suggests that the numbers of viruses in the waters were exceptionally high. No viruses were detected in the stream upstream of the settlement. Viruses outnumbered faecal bacteria and phages commonly used as indicators in a substantial number of stream water samples. This confirms the exceptional high incidence of viruses in the waters concerned as well as shortcomings of faecal bacteria and phages commonly used as indicators of water quality. The high incidence of all three vaccine strains of polioviruses in the absence of wild-type strains, confirms the success of poliomyelitis vaccination in the communities concerned. The results show that diffuse effluents from the settlement heavily pollute the stream with viruses and other faecal organisms. This has major implications for risks of infection constituted by the stream. The findings underline the need for efficient sanitary services in communities of this kind in order to protect water sources and to control waterborne diseases.

INTRODUCTION

Viruses are excreted in large numbers by infected individuals and may remain infectious for days, weeks or months in water environments (Grabow, 1996). In addition, the minimum infectious dose of viruses may be as low as a single particle. Viruses are, therefore, a common cause of waterborne disease. Details on the incidence and behaviour of viruses in water environments are limited because their enumeration requires advanced technology, and commonly used faecal bacteria have shortcomings as indicators for viruses. Details on viruses in waste water are essential for health risk assessment and management strategies aimed at the protection of water sources and the control of waterborne diseases. Virological screening of wastewater is also used for other purposes such as the investigation of enteric viruses circulating in communities, and monitoring of public health campaigns such as poliomyelitis vaccination (Tambini *et al*, 1993; Van der Avoort *et al*, 1995). This study focuses on viruses in diffuse effluents from a typical low socio-economic informal settlement with restricted sanitation and water supply. These communities are exceptionally vulnerable to enteric infections (Von Schirnding *et al*, 1993), and waste water effluents are likely to contain high levels of faecal organisms.

MATERIALS AND METHODS

A total of 209 samples were collected over a period of 9 months in 1994 and analysed for enteric viruses and related indicators. These samples were collected at regular intervals from a stream which runs through the settlement and a sewerage pipeline which serves an adjacent community. The stream was sampled about 3 km upstream of the settlement, right in the settlement and about 1 km downstream of the settlement. Cytopathogenic viruses were isolated from unconcentrated samples by conventional cell culture propagation using the BGM monkey kidney and PLC/PRF/5 human liver cell lines, and primary vervet kidney cells (Grabow and Taylor, 1993). Faecal coliform bacteria, enterococci, somatic and male-specific coliphages, and phages infecting *Bacteroides fragilis* HSP40 were enumerated by conventional methods (Grabow, 1996). Viruses were typed by cytopathogenic effect (CPE) in cell cultures and stained cover slips, inoculation of newborn mice, neutralisation tests using the Lim Benyesh-Melnick antiserum pools for enteroviruses, and molecular techniques using routine procedures (Grabow *et al*, 1992). The National Institute for Virology, Johannesburg, kindly rendered assistance in the typing of certain isolates, notably the confirmation of vaccine strains of polioviruses. Adenovirus typing was limited to distinction between enteric and non-enteric types using the commercial adenoclone-type 40/41 immunoassay (Cambridge Biotech, Worcester, MA).

RESULTS

No viruses were detected in samples of water from the stream upstream of the settlement. In these samples counts of faecal coliforms were in the range of 100 to 2800 per 100 ml. Somatic and male-specific coliphages were detected only on rare occasions, and counts were in the range of 0 to 200 per 100 ml. A total of 486 viruses were isolated from 72% of 142 samples of stream water collected in and below the settlement, and from 96% of 53 sewage samples. The isolates consisted of 263 coxsackie B viruses (54%), 109 polioviruses (22%), 9 adenoviruses (2%), 2 echoviruses (0,3%) and 2 reoviruses (0,4%). The remaining isolates consisted of 42 viruses only typed as enteroviruses (9%), and 59 only typed as enteroviruses other than coxsackie- or polioviruses (12%). All six types of coxsackie B viruses were isolated, but 48% were coxsackie B2. All three types of polioviruses were isolated. All the poliovirus isolates were vaccine strains. All 9 adenovirus isolates were non-enteric types. Details on viruses isolated from the stream and sewage samples are jointly summarised in Table 1, because the relative proportions of various viruses isolated from the stream and sewage did not differ significantly. Table 2 compares the percentages of viruses isolated on each of the three cell culture types, which gives an indication of the efficiency of the cells for the isolation of viruses from environmental waters. Many of the viruses only yielded a CPE after two or three blind passages.

Maximum levels of faecal coliforms, enterococci, somatic coliphages and male-specific (F-RNA) coliphages are recorded in Table 3. These results show that maximum levels of enterococci and coliphages in the stream were close to those in sewage. Counts of indicator organisms and the incidence of viruses tended to increase after rainfall, which shows that stormwater run-off contained heavy loads of faecal excreta. The following numbers of samples had counts of faecal bacteria or phages lower than those of viruses: faecal coliforms (2 samples), enterococci (14 samples), somatic coliphages (4 samples), male-specific coliphages (25 samples) and *B fragilis* HSP40 phages (many samples). The latter number of samples is not clearly defined because low counts of phages could at times have been due to malfunctioning of the phage test. Counts of faecal bacteria and phages were lower in the stream at the sampling site 1 km downstream of the settlement. In this area the stream slowly passed through some reed-beds and stagnant patches of water which clearly reduced counts of the organisms. The percentage of samples from which viruses were isolated was, however, the same (72%) for samples collected from the stream inside and downstream of the settlement, suggesting no significant reduction in numbers of viruses.

Table 1. Viruses isolated from 142 samples of polluted stream water and 53 samples of sewage

Virus	Isolates on each cell culture			Total
	PLC	BGM	VK	
Coxsackie B1	9	9	1	19
Coxsackie B2	47	43	37	127
Coxsackie B3	16	10	14	40
Coxsackie B4	16	26	18	60
Coxsackie B5	2	0	1	3
Coxsackie B6	3	5	6	14
Total coxsackie B	93	93	77	263
Polio Sabin 1	39	26	15	80
Polio Sabin 2	6	10	2	18
Polio Sabin 3	6	2	3	11
Total polio	51	38	20	109
Echo 7	0	0	1	1
Echo 27	0	0	1	1
Entero non-cox/polio	22	24	13	59
Entero untyped	10	17	15	42
Adeno	9	0	0	9
Reo	0	0	2	2
Total isolates	185	172	129	486

Samples collected from stream inside settlement and about 1 km downstream.

Entero non-cox/polio = enteroviruses other than coxsackie or polio.

Entero untyped = enteroviruses not typable by available neutralising typing antisera.

Relative composition of viruses isolated from the stream polluted by diffuse effluents from the informal settlement did not differ significantly from that of sewage of an adjacent community.

Table 2. Comparison of three cell culture types for the isolation of cytopathogenic viruses from a polluted stream and sewage in developing communities

Virus	Percentage isolates on each cell culture			Percentage of total
	PLC	BGM	VK	
Coxsackie B1	47	47	6	4
Coxsackie B2	37	34	29	26
Coxsackie B3	40	25	35	9
Coxsackie B4	27	43	30	13
Coxsackie B5	67	0	33	1
Coxsackie B6	21	36	43	3
Total coxsackie B	35	35	30	54
Polio Sabin 1	49	33	18	17
Polio Sabin 2	33	56	11	1
Polio Sabin 3	55	18	27	2
Total polio	41	38	21	22
Echo 7	0	0	100	0,3
Echo 27	0	0	100	0,3
Entero non-cox/polio	37	41	22	12
Entero untyped	24	40	36	9
Adeno	100	0	0	2
Reo	0	0	100	0,4
Total isolates	38	35	27	100

Total number of isolates = 486.

Percentages calculated from numbers of isolates presented in Table 1.

PLC = PLC/PRF/5 human liver cell line.

BGM = Buffalo Green Monkey kidney cell line.

VK = Primary vervet kidney cells.

Table 3. Maximum levels of faecal bacteria and coliphages in the stream and sewage

Indicator	Count/ml	
	Stream	Sewage
Faecal coliforms	733 000	30 000 000
Enterococci	33 300	62 000
Somatic coliphages	20 000	22 500
F-RNA coliphages	1016	1200

DISCUSSION

This study represents the most comprehensive survey of viruses in water recorded to date in South Africa, and probably the most detailed investigation of viruses in diffuse effluents from an informal settlement anywhere in the world. Although viruses have not been enumerated, the high percentage of samples from which viruses were isolated without the application of recovery techniques suggests that the incidence of viruses in the stream downstream of the settlement as well as the sewage were exceptionally high compared with data on the general incidence of viruses in sewage and waste water, according to which numbers of cytopathogenic viruses are generally about 200 to 11 000 per litre, but may exceed 100 000 per litre (WHO Scientific Group, 1979; Dahling *et al*, 1989). The viruses isolated in this study are limited to viruses which cause a cytopathogenic effect in cell cultures. These viruses probably only represent the tip of the iceberg of the total number of viruses in the waters because the great majority of viruses concerned are not detectable by cell culture propagation (Bern and Glass, 1994; Grabow, 1996).

The finding that viruses outnumbered faecal coliforms, enterococci and all three phages in a substantial number of river water samples, confirms the exceptionally high numbers of viruses, as well as shortcomings of commonly used indicators for indicating the presence of viruses. The high proportion of coxsackie B viruses (54% of all isolates, Table 2) is in agreement with results reported for sewage in other parts of the world (Dahling *et al*, 1989). The finding that the three vaccine strains of polioviruses represented 22% of all viruses isolated (Table 2), in the absence of wild type strains, confirms the success of poliomyelitis vaccination campaigns in the communities concerned and indicates that the risk of poliomyelitis in the communities is low.

A total of 101 isolates (Table 1) have only been typed as far as enteroviruses, or enteroviruses other than coxsackie or polio. These isolates probably belong to the wide range of 33 different serological types of echoviruses. This has been confirmed by typing two of the isolates in this group, which turned out to be echovirus types 7 and 27. Typing of these viruses is extremely labour intensive and expensive, and contributes little to the objectives of this particular study. The nine isolates of adenoviruses (Table 1) were likewise not typed any further than confirming that they were not enteric adenoviruses, ie types 40 or 41. These adenoviruses do, therefore, belong to any of the remaining 44 serological types.

The PLC/PRF/5 cell line proved most useful for the isolation of viruses from environmental waters because 38% of viruses were isolated on these cells, compared with 35% on BGM cells and 27% on primary vervet kidney (VK) cells (Table 2). PLC/PRF/5 cells were particularly susceptible to polioviruses, and all 9 adenoviruses were isolated on these cells. The susceptibility of PLC/PRF/5 cells to adenoviruses would seem to be in agreement with their ability to support the replication of enteric adenoviruses (Grabow *et al*, 1992). However, primary vervet kidney cells would seem to be more susceptible to echoviruses and reoviruses than the other two cell types. In combination the data on viruses isolated (Table 2) suggest that ideally all three cell types should be applied in parallel for the isolation of viruses from environmental waters. In a previous comprehensive evaluation of a wide variety of cell culture systems, the three cell culture types used in this study have been selected as the most sensitive for the isolation of viruses from environmental waters (Potgieter and Grabow, 1995). The low number of reoviruses isolated in this study would appear surprising because relatively higher numbers of reoviruses were recorded in studies on other waters (Grabow and Nupen, 1981; Dahling *et al*, 1989). This may imply variation in the incidence of reoviruses because the cell cultures and conditions of viral propagation used in this study are ideally suited for the isolation of reoviruses.

This study shows that diffuse effluents from the settlement heavily polluted the stream with viruses and other faecal organisms. At peak levels of pollution, particularly when stormwater run-off from the settlement entered the stream, counts of enterococci and phages in the stream were close to those in raw sewage (Table 3). These observations have major implications for the downstream utilisation of the water in the stream, because the quality by far exceeded levels considered acceptable for the utilisation of water for domestic purposes, recreation and irrigation. Since the study site was selected as typical of similar situations elsewhere, the results underline the importance of appropriate sanitary services for informal settlements.

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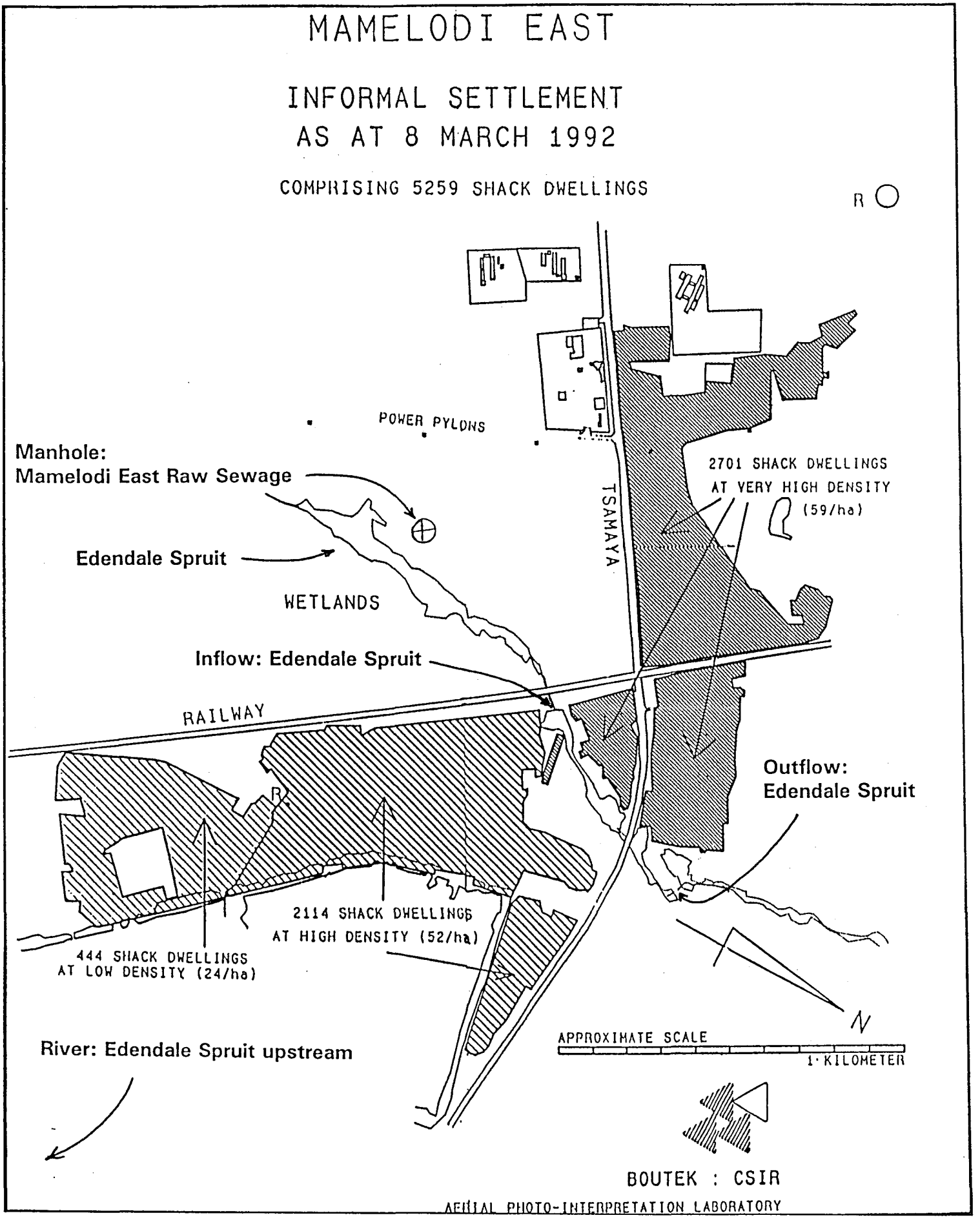
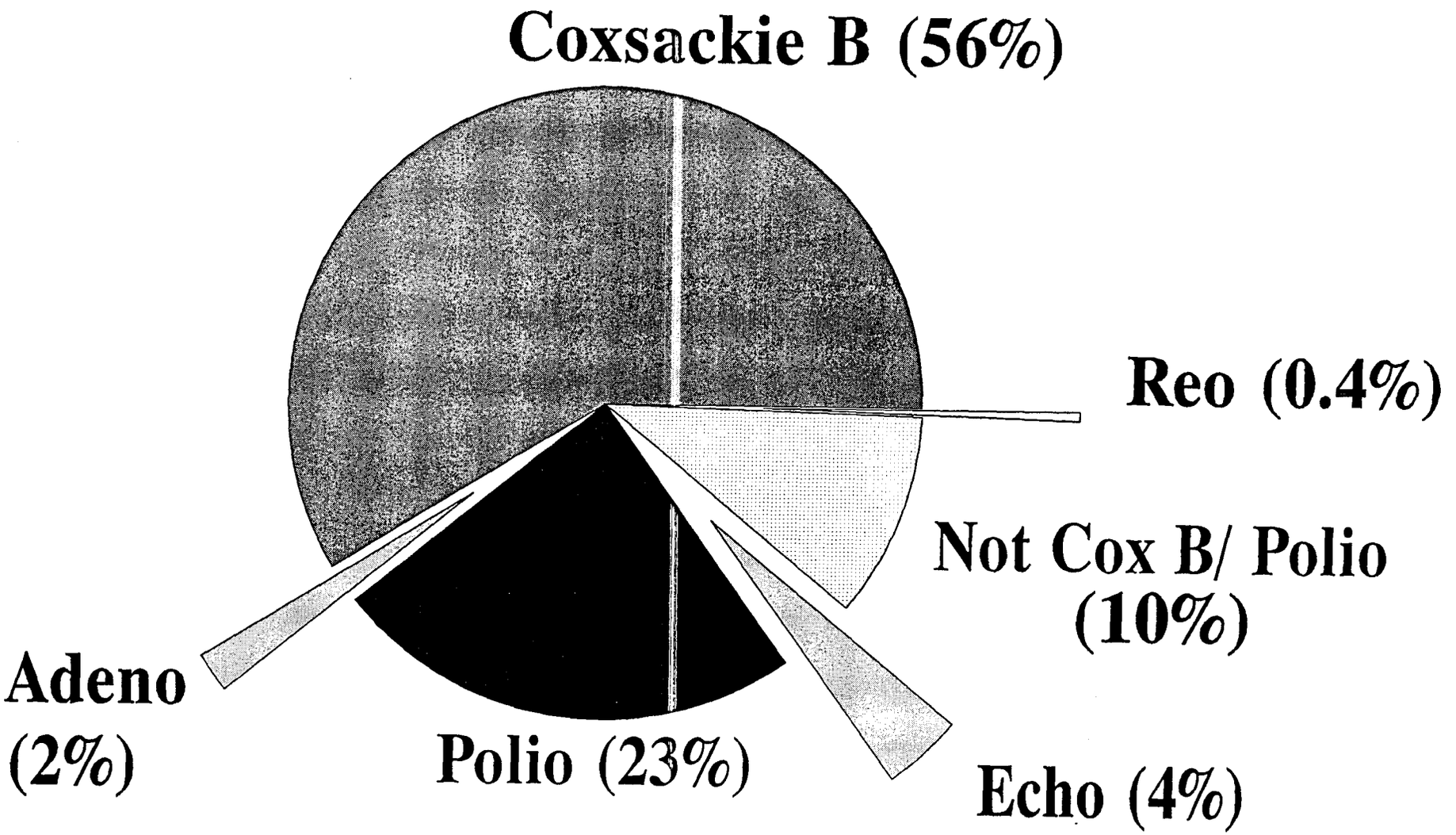
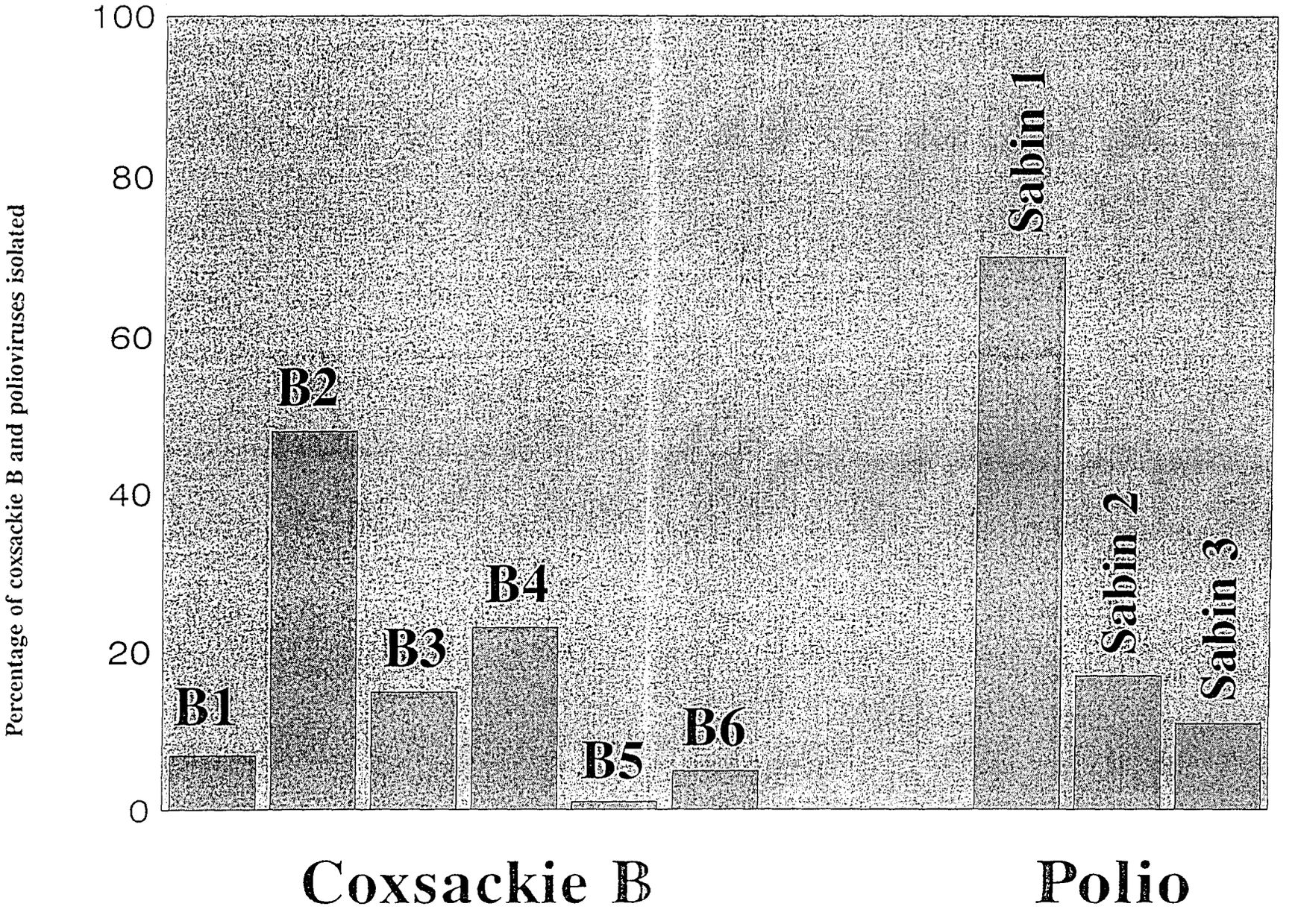


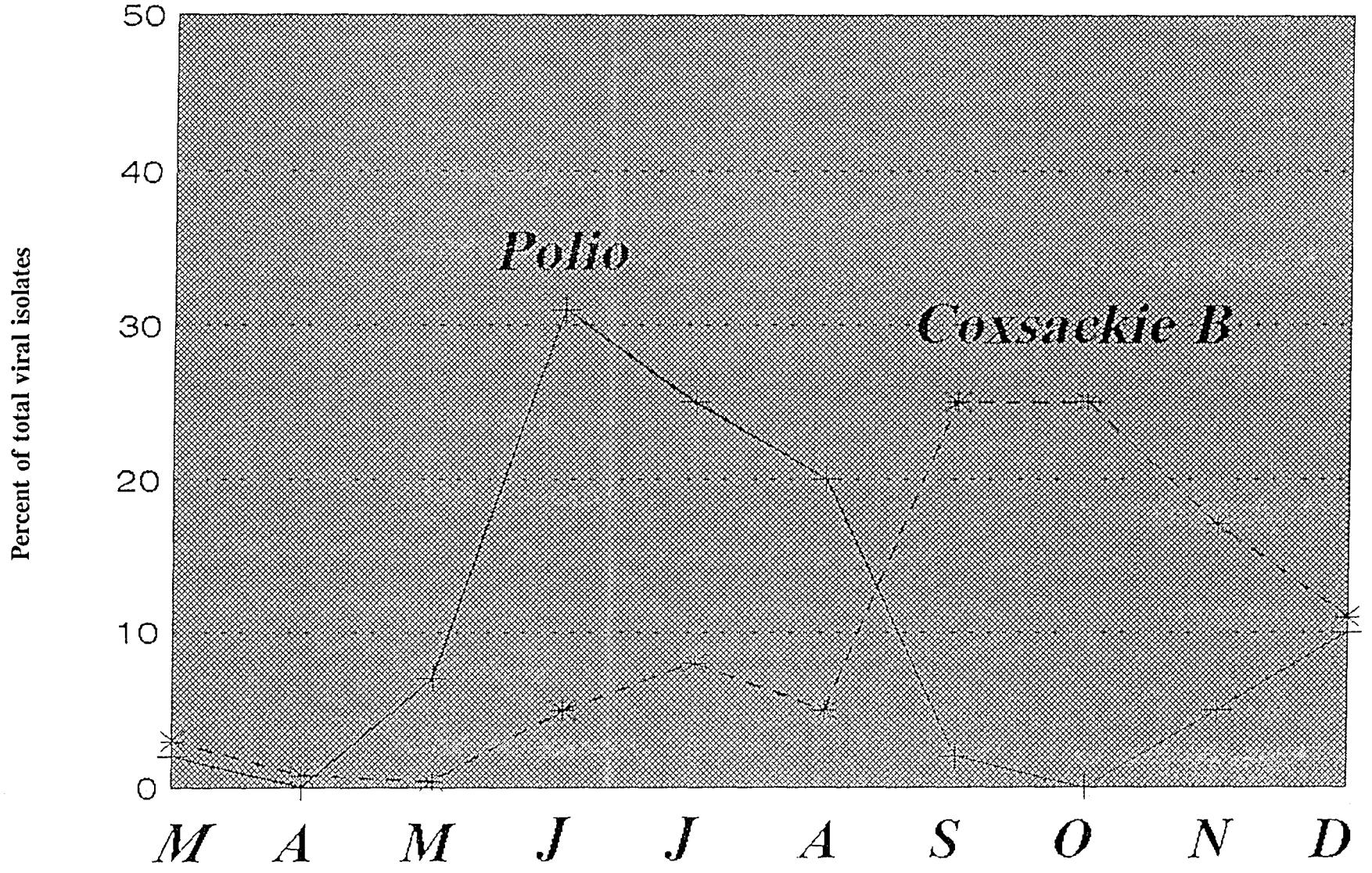
Map of study area and sampling sites



Types of viruses isolated from sewage and the Edendale Spruit in the study area



Percentage of individual strains of coxsackie B and polioviruses isolated



Percentage of polio and coxsackie B viruses isolated from March to December 1994