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Detection of water-borne pathogens: culture plate to genomics

Usha Dixit[1] and Rishi Shanker[2]
[1]*Science & Society Division, DST, Technology Bhawan, New Mehrauli Road, New Delhi-110016, India*
[2] *Industrial Toxicology Research Centre, P.O. Box 80, M.G. Marg, Lucknow-226001, India*
usha.dixit@nic.in

Abstract: This paper describes the use of different analytical techniques for detection of water borne pathogens. It discusses the brief information about the development of new diagnostic and monitoring techniques available and explains its important role in risk management of water related diseases. New techniques will aid development of strong early warning systems, reliable field diagnostics, treatments and more effective remediation of impacts from harmful microorganisms. The paper proposes that the future developments in signal detection and miniaturization technologies will provide real-time monitoring and diagnostics for rapid assessment of microbial pathogens.

Keywords: Water-borne pathogens, risk management, microbial indicators, real-time monitoring, health.

Introduction

Water pollution seems to be inevitable consequence of urbanization and industrialization in developing and developed countries. Water resources are overburdened due to contamination from a) sewage and pathogenic agents, b) industrial and trade wastes, c) agricultural pollutants, like fertilizers and pesticides, and d) thermal pollution. Water borne diseases have a major public health and socio-economic impact. WHO/UNICEF Report estimates that over 2 million people die annually due to water related diseases (WHO, 2000).

Water may harbor pathogenic bacteria, viruses, protozoa and parasites responsible for the emerging most widespread infections which are leading cause of death worldwide. These pathogens are diverse in nature placing entire communities at risk (Sarkar *et al*.,1999). An outbreak of cholera caused by *Vibrio cholerae* was recorded in western Kenya between June1997-March 1998 (Shapiro *et al*.,1999). In June 1998 hundreds of children playing in water theme park in suburban Atlanta were exposed to *Escherichia coli* due to faecal contamination and insufficient chlorine levels (Rose & Grimes, 2001). Lemus and Weisburg (2000) reported beach closures in 1999, in Huntington Beach, California due to high levels of enterococci and coliform bacteria. Infantile diarrhea due to entropathogenic *Escherichia coli* has been reported in Delhi and Calcutta in 1997-1999 (Prasannan *et al*., 2001). A study carried out in various countries in Asia identified enteroaggregative and enterotoxicogenic *Escherichia coli* as the pathogens responsible for acute/persistent diarrhea in India (Black, 1993). Also concurrent outbreaks of cholera have been reported in Gujarat, Orissa and Kerala, India in the year 2000 with new locations such as the Kottayam district in Kerala (Samal *et al*., 2001; Chakraborty *et al*., 2001). In Japan 1993, enteroaggregative *Escherichia coli* caused massive outbreak of gastrointestinal illness in almost 2,700 school children (Itoh *et al*., 1997), while enteroaggregative *Escherichia coli* caused outbreaks of gastroenteritis in U.K. (Smith *et al*., 1997). In 1995-1996, the largest outbreak of infection caused by SF STEC 0157:H7 resulting in Hemolytic Uremic Syndrome and diarrhea was reported in Bavaria, Germany (Ammon *et al*., 1999). In 1993, municipal water supply contaminated with *Cryptosporidium* spp. caused largest recognized outbreak in Milwaukee of United States (McDonald *et al*., 2001). Two *Giardia* outbreaks has been reported in Florida in 1998 caused from untreated ground water (MMWR, 2000). The outbreak of gastroenteritis in May 2000, was predominantly caused by *Escherichia coli* 0157:H7 and *Campylobacter* in Walkerton, Ontario of Canada (Canada Communicable Disease Report (2000).

Regulation and risk assessment

Health based targets provide the basis for the application of the National and International Standards for drinking water quality for community and household supplies. Water quality guidelines and standards, recommended by various authorities, might be similar to ensure the minimum risk of infection (Table1), but differ due to economic and technical capabilities and perceptions of acceptable risks of infections in rural and urban environment (Fig.1). Guidelines are intended to support the development and implementation of risk management strategies to ensure the safety of drinking water supplies. To date there are international agreements to legal limits for pathogens as reflected by EU directives and WHO's international quality safety standards (Table 1).

A safe supply of drinking water depends upon use of either protected water sources, or properly selected and operated series of interventions, use of appropriate treatment technologies capable of reducing pathogens and other contaminants and finally prevention of re-contamination in distribution (Fig.1). Characterization of water quality of a resource is carried out, to reduce the health hazards due to water-borne diseases, to acceptable levels. Disinfection is of unquestionable importance in the supply of safe drinking water and is an effective barrier to many pathogens in drinking water. It involves the use of reactive chemical agents such as chlorine which can be easily monitored and controlled as a drinking water disinfectant. Protection of the source and treatment techniques such as chlorination and efficient filtration has been recommended to ensure absence of

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Table 1. Bacterial contamination regulations and guidelines for drinking water

Country	Total coliform	*E. coli*
India (BIS)	10/100ml (95% should not contain any coliform organisms in 100ml)	0/100ml (100%)
USA	0/100ml (95%), a consecutive sample from the same site must be coliform free /, <5% positive	0/100ml (100%)
Canada	0/100ml (90%) none should contain more than 10 CFU/100ml,a consecutive sample from the same site must be coli form free	0/100ml (100%)
EEC	0/100ml, or MPN<1	0/100ml (100%)
IS 10500	0/100ml	0/100ml (100%)
WHO	0/100ml(95%)	0/100ml (100%)
OECD	0/100ml	0/100ml (100%)
UK	0/100ml(95%)	0/100ml (95%)
EC	0/100ml	0/100ml

(Ref: Parsons, 2000; Balis et al., 1996; Houndt & Ochman, 2000; Bhanumathi et al., 2003; Karlowsky et al.,2003)

viruses, protozoas and helminths due to lack of routine monitoring techniques (WHO, 2003). Chlorine disinfection has limitations against protozoans, some viruses or pathogens within flocks or particles. It has become necessary to treat sewage water to meet objectives for drinking water in densely populated areas. Soon densely populated cities may have to treat urban storm water and wet water overflows to conserve and over come water scarcity. Therefore, it is essential that an overall management strategy is implemented, where multiple barriers as well as, protection in distribution are used in conjunction with disinfection, to prevent or remove microbial contamination.

OECD's expert working group examined approaches for establishing links between drinking water and infectious diseases, and new approaches to enhance current methods for surveillance and outbreak investigation, in particular the development of real-time measurements and predictive models. The World Health Organization, the Pan American Health Organization and UNESCO held an expert consultation in Mexico City in 1997 on *Entamoeba histolytica* and *Entamoeba dispar* for developing techniques for differentiating between the two protozoa during diagnosis, identifying virulence factors and developing immunological studies to determine the feasibility of a vaccine (CRC for Water Quality and Treament, 2000).

The problem of detection and monitoring of microbial pathogens in drinking water is being viewed in a global perspective (Fig. 2). A recent United Nation's report portrays a grim picture of water quality conditions in some developing countries (WHO, 1997). Yet common surveillance tools for water borne pathogens are needed to reduce the risk by standardizing methodologies and validation on international level. Risk assessment to address the risk of exposure and the potential health impacts has been carried out (WHO, 1997). However, all of the microorganisms currently on EPAs "Contaminant List" lack database resulting in the failure on risk assessment on exposure. Thus, accurate and preemptive monitoring information will provide advances in the microbial risk assessment (Fig.3).

Regulatory frame work must be encouraged for the use of new cost-effective technologies with comprehensive efficacy which will assist water utilities for handling data and interpreting sensitive data, thereby protecting the environment as well as human health. e.g.-

-Policy makers to be educated on research, development and availability of new technologies

- Problems requiring risk assessment

-Voluntary monitoring of activities by water utilities

-International collaboration for evaluation of new methods

-Availability of data base for generation of guidelines and recommendation

Thus, the regulatory requirements are demanding and represent a significant challenge for new technologies. New techniques will aid development of strong early warning systems, reliable field diagnostics, symptom treatments and more effective remediation of impacts from harmful microorganisms.

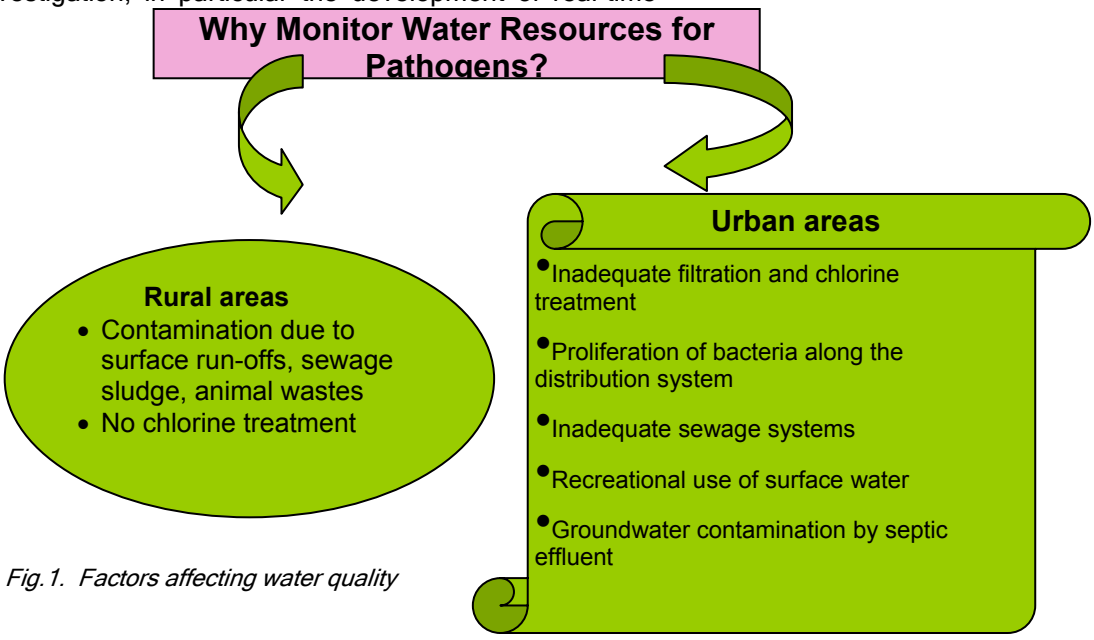

Fig.1. Factors affecting water quality

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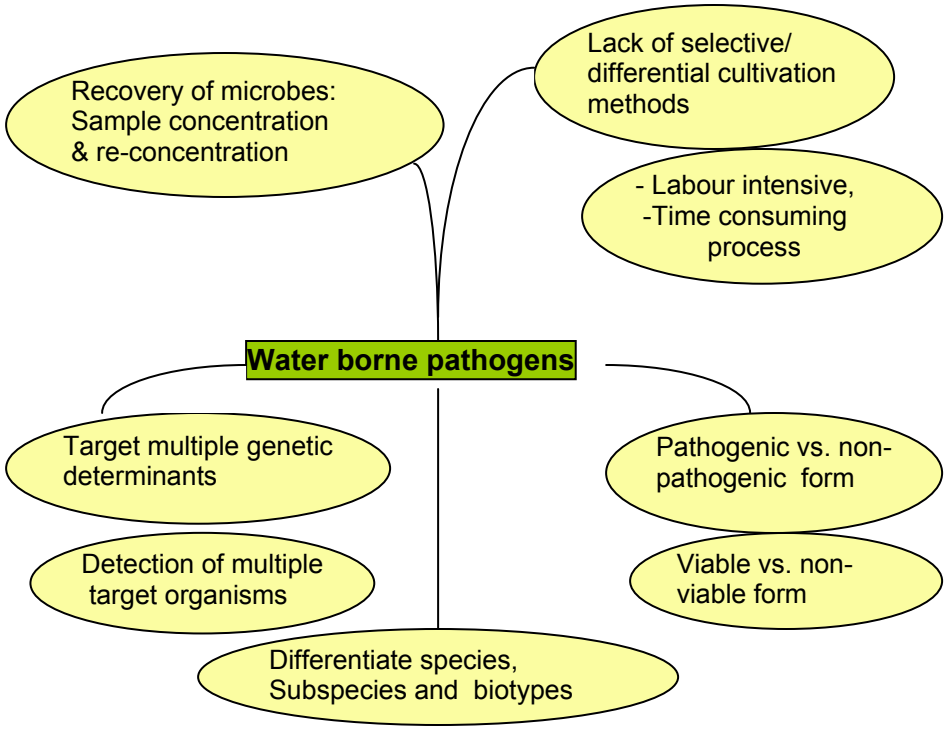
Fig. 2. Limitations in detection and monitoring of water-borne pathogens



'Indicator' microorganisms and 'Marker' pathogens

Sources of water pollution must be identified in order to adequate water quality problems and protect public health. Source detection provides direct evidence of the origin of pollution by identifying indicator organisms. Indicator microorganisms are used to predict the potential risk from pathogenic microorganisms and circumvent the need to assess every pathogen. Few criteria have been recognized for the selection of indicator organisms (Payment, 1998):e.g.-

-should be associated and present in larger numbers than pathogen

-should survive equally or longer than pathogen in environment

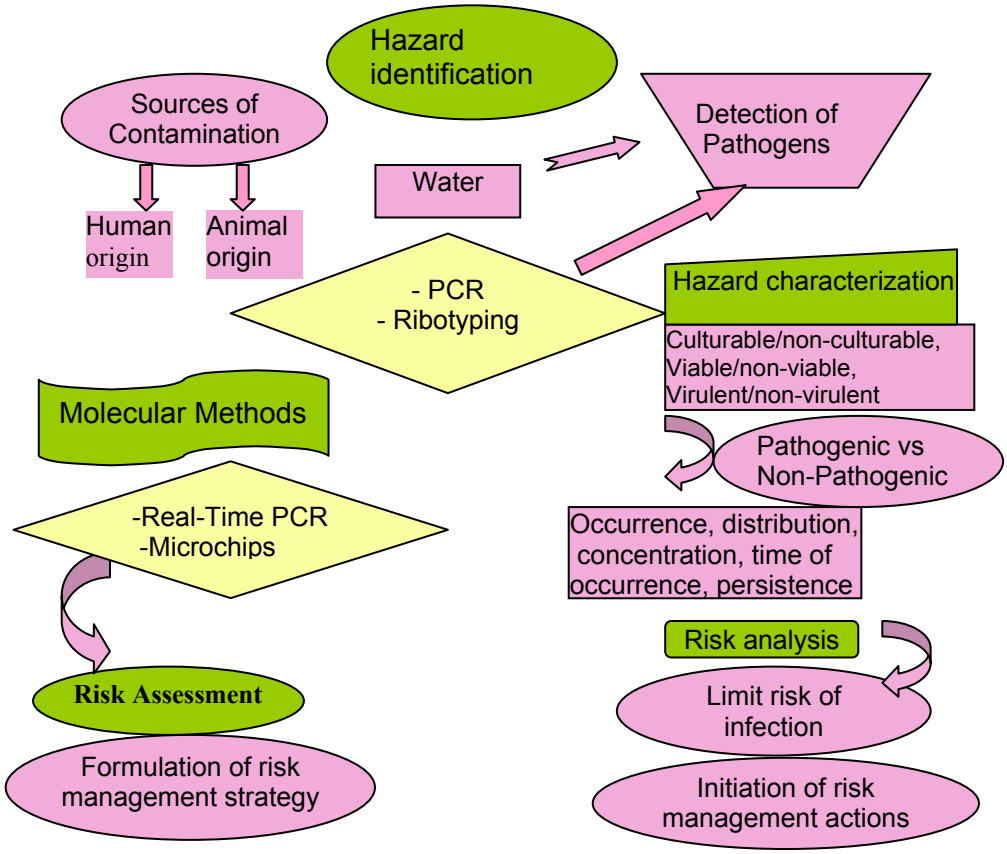
-should have stable characteristics and give consistent reactions in analyses

-should have resistance to treatment equal or greater than pathogen

-should be easy to enumerate

Indicators are needed for the reason being- failure of expensive, time consuming difficult reactive process to provide warning, are easier to detect and to assess water quality (Toranzos & McFeters, 1997). Therefore, a system is required for identification of water supplies, before the occurrence of disease outbreak. However, due to ubiquitous nature of microorganisms, the effectiveness of traditional indicators to predict the presence of human or animal waste impact and subsequent health risk is limited. Often epidemiologic studies fail to show any relationship to microbial indicators, due to poor design and/or due to the widely fluctuating ratio of pathogen(s) to faecal indicators and the varying virulence of the pathogens. The validity of any indicator system is also affected by the relative rates of

Fig. 3. Role of molecular methods in identification and assessment of water-borne pathogens



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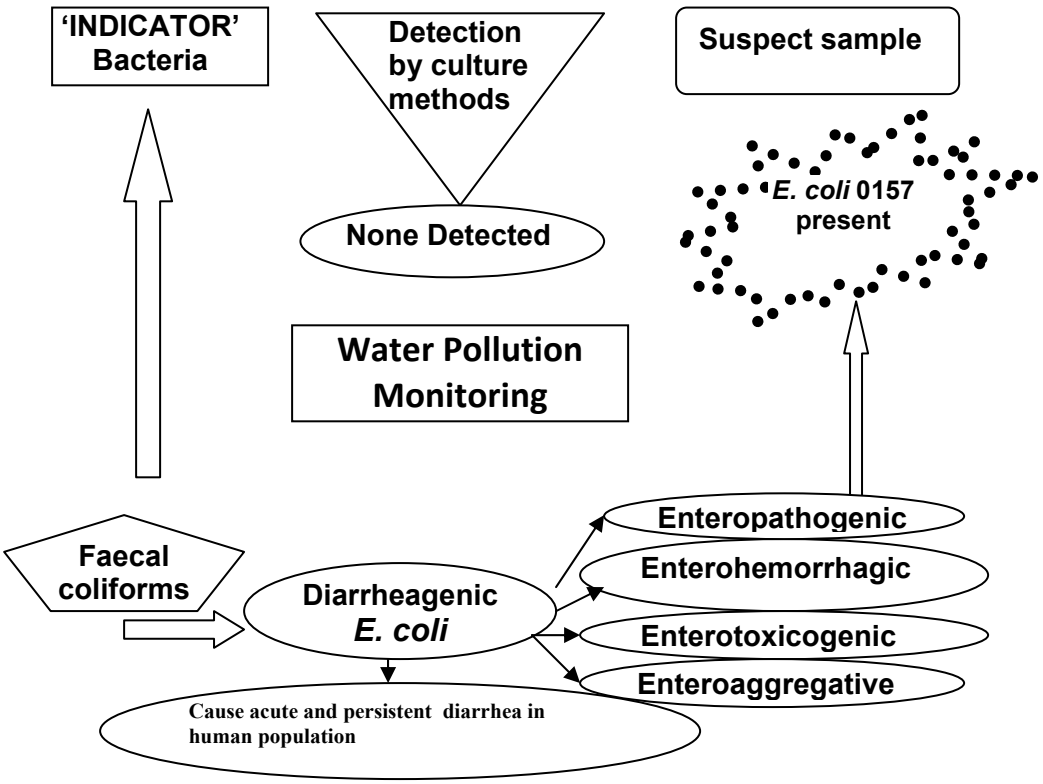
removal and destruction of the indicator versus the target hazard. So differences due to environmental resistance

Fig. 4. A schematic presentation of discrepancy of culture methods to detect evolving pathogenic 'indicator' biotypes



or even ability to multiply in the environment, all influence their usefulness. Indicators have traditionally played a very important role in guidelines and national standards. Increasingly, however, they are being seen as an adjunct to management controls, such as sanitary surveys, and there is a move away from a specified indicator level end product. Hence, there is a need globally to identify 'marker' organisms or pathogens in geographically specific water resources. It is necessary to define reference populations of pathogens to focus on specific sensitive groups in deriving national standards. Several indicators (Fig. 4, Table 2) have been studied and recommended for water quality assessment (ISO,1990; Standard Methods,1992; Guyot *et al*., 2002) which include bacteria (*Escherichia coli, Salmonella, Shigella, Vibrio, Pseudomonas*), viruses (*Enteroviruses, Rotaviruses, Hepatitis A and E*), protozoa (*Entamoeba, Giardia, Cryptosporidium*).

Genomics of water-borne pathogens

Bacterial genomes are composed of universally present conserved 'core' genes providing backbone of genetic information. The genome also contains a flexible gene pool consisting of an assortment of strain specific genetic information which may provide additional capabilities enabling the adaptation of the species to environmental conditions. This difference in genome size reflects size variations of the flexible gene pool due to the acquisition and loss of genomic DNA (Dobrindt & Hacker, 2001). Bacterial adaptation has been influenced by horizontal gene transfer (HGT). HGT has led to changes in microbial genomes over relatively short time periods and is responsible for ubiquitous occurrence of pathogens. The dissemination of antibiotic resistance genes among human and non-human pathogens is the paradigm for HGT on a global scale (Mazel & Davies, 1999). Studies of antibiotic resistance development are essentially retrospective but less information is available for microbial dynamics of this process. Besides, plasmid and bacteriophages, large genome regions (up to 40 Kb) known as pathogenicity islands (PAIs) are consistently present in pathogenic strains carry many virulence genes. The sequenced *Helicobacter pylori* strain contains a single contiguous "cag" PAIs (Censini *et al*., 1996). PAIs has also been reported in *Salmonella typhimurium* encoding typeIII secretion systems during infection (Shea *et al*., 1996). Pathogenicity islands has been identified in *Escherichia coli* isolates (PAIs I, II, IV, V, *hlyI*, *hlyII, kps, pap, sfa, prf*) (Boyd & Hartl, 1998). Also STEC 0157:H7 strains has been shown to harbour a TAI which encodes for a novel adherence-confering protein and tellurite resistance (Tarr *et al*., 2000). The deletion of PAIs from the chromosome or the acquisition by other species or genera may lead to new pathogenic variants showing flexibility and evolution of microbial genomes.

Virulence in pathogens is often multifactorial and coordinately regulated and virulence genes tend to be clustered in the genome. Numerous phages carrying virulence determinants in *Pseudomonas aeruginosa, Vibrio cholerae*, *Shigella dysenteriae* and *Escherichia coli* have been identified (Hacker *et al*.,1997). Salama *et al.* (2000) and Israel *et al.* (2001) have reported association of specific genes with Virulence Island in *Helicobacter pylori* with increase in pathogenicity. A study on the association between known virulence factors of shiga toxin producing *Escherichia coli* revealed that *stx2* positive isolate is approximately five times more likely to be associated with severe disease in humans than a *stx2*

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negative isolate of the same serotype. Genome of *Escherichia coli* 0157:H7 sequenced so far indicates more than 20 potential virulence genes clustered in several mobile genetic elements (Cruz & Davies, 2000). Mobile genetic elements such as plasmids and transposons can effectively circumvent the genetic barriers between bacterial species by conjugation and transductional transfer of DNA molecules. For example, the virulence plasmids of *Yersinia, Shigella* and *Escherichia coli* and temperate phages carrying virulence determinants in *Pseudomonas aeruginosa, Vibrio cholerae, Shigella dyenteriae* and *Escherichia coli* (Censini *et al.,*1996; Salama *et al.,* 2000; Israel *et al.,* 2001).

Table 2. Certain microorganisms present in water resources

Organism	Genome* size	Pathogenicity	Antibiotic Resistance	References
E. coli	5.2Mb	Diarrhea, haemorragic colitis (HC), hemolytic uraemic syndrome (HUS)	Ampicillin, chloramphenicol, kanamycin, tetracycline, protamine	Balis *et al.*,1996; Houndt & Ochman, 2000
V. cholreae	4.0Mb	Cholera	Ampicillin., ciprofloxacin, streptomycin, pteridine, sulfamethoxazole, trimethoprim	Bhanumathi *et al.*,2003
P. aeruginosa	6.3Mb	Septicaemia, meningitis, nosocomial pneumonia &UTI, wound infection	Ampicillin, chloramphenicol, streptomycin, tetracycline, piperacillin, piperacillin-tazobactam	Karlowsky *et al.*, 2003
Shigella	4.59Mb	Abdominal cramps, fever, watery diarrhea	Ampicillin, chloramphenicol, tetracycline, trimethoprim, sulfamethoxazole	MMWR, 2000
Salmonella	4.85Mb	Gastroentritis, bacterimia, septicaemia, enteric fever, intestinal ulcer	Ampicillin, chloramphenicol, kanamycin, tetracycline, Protamine	Balis *et al.*,1996
E. faecalis	3.36Mb	Endocarditis, biliary tract infection, septicaemia, peritonitis, intra abdominal abscess	Penicillin, vancomycin, ampicillin, erythromycin, tetracycline	Haque, *et al.*,2000
H. pylori	1.66Mb	Chronic gastritis, peptic and duodenal ulcers, gastric cancer	Vancomycin, trimethoprim, polymixin B, tinidazole, amoxycillin, clarithromycin	Grabow, 2002; Belzer *et al.*, 2009
E. histolytica	20Mb	Dysentry colitis, amoeboma	Metronidazole	APHA,1992
Enteric viruses Polio, Coxsackie, Hepatitis A & E, Rota virus	7176-7478 bp	Gastroentritis, respiratory disease, meningitis, paralysis, Hepatitis, conjunctivitis, Diarrhea, Gastroentritis	Antviral-interferon α, zidovudine, didanosine	Hijnen *et al.*, 2000

**Source: TIGR microbial database (www.tigr.org)*

Recently, the existence of a complementary but inverse pathway enabling the bacteria to evolve towards a pathogenic lifestyle: the formation of "black holes" i.e. deletion of genes that are detrimental to a pathogenic lifestyle has been reported (Souza & Eguiarte, 1997). This mechanism represents a subtle fine-tuning of the genome repertoire of created pathogen to delete the genes detrimental to pathogenic lifestyle. Black holes can precede addition of PAIs enhancing the expression of virulence genes. Maurelli *et al.* (1998) have reported deletion of cadA of *Shigella* spp and EIEC representing a black hole in the genome which promotes the virulence of these pathogens. Occurrence of pathogenic *Shigella* species from a non pathogenic *Escherichia coli* ancestor,by the loss of the ompT and cadA (black hole) genes are known to contribute to their evolution (Nakata *et al.,*1993; Hurtado & Rodriguez-Valera, 1999). The formation of 'black holes' for enhancement of virulence expression is a possibility to study natural inhibitors of virulence in the treatment of infectious diseases.

Pathogen populations exhibit diversity as a result of the presence of diverse genotypes, or genetically identical members changing the expression state of certain genes as in *Escherichia coli* (Hayashi *et al.,*2001). Genomics in infectious diseases is helpful in understanding the phylogenetic diversity of pathogens and evolution of pathogenicity, the development of new preventive and therapeutic measures and the implementation of new diagnostic procedures for the detection and typing of pathogens. Pathogen diversity can be used to develop models of the evolution of pathogen and pathogenicity which will help in sophistication of public health interventions.

Comparative and functional genomics is a powerful approach towards understanding mechanisms of microbial pathogenesis and reveals new insights into bacterial evolution and the diversity of microbial pathogens. Therefore comparative genomic analysis is useful for micro organisms for which traditional genetic techniques are difficult or impractical, genomic sequence can be used to make biological predictions of these

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Table 3. Methods used for the detection of waterborne pathogens

Technique	Organism	Advantages	Reference
Phenotypic			
Faecal coliform/faecal streptococcus ratio	Coliforms	Rapid results with minimal expertise	Edberg *et al.*,1988
Culture Methods	*E. coli, E.faecalis,* Coliform	Determination of concentration of microorganisms	APHA, AWWA, AEF,1998
MTF, MF	Coliforms, *E. coli*	Inexpensive, easy to implement and perform	Rompere *et al.*, 2002; Versalovic & Lupskin, 2002
PA	*E. coli, Pseudomonas sp*	Inexpensive, rapid and sensitive quantitative detection	Mandrell & Wachtel, 1999
MAR	*E. coli, H. pylori, Enterococcus*	Detection of genetic determinants conferring resistance for different antibiotics	Osaki *et al.*,1998.
Immuno-magnetic separation	*E. coli, H. pylori*	Increased assay speed, concentration of target micro organism, Recovery of sub lethally damaged cell	Maurer *et al.*,1999; Eaton & Gasson, 2001; Fu *et al.*, 2005.
Serotyping	*E. coli, V. cholerae, Shigella, Salmonella, P.aeruginosa*	Differentiation of microorganism from various sources	Vandamme *et al.*,1995.
Genotypic			
PCR	*E.coli, E. faecalis, Shigella, H. pylori, S.,Vibrio cholerae, E. histolytica P. aeruginosa, Cryptosporidium*	Detection of specific infective agents and their virulent genes Identification of functionality of genetic element, Rapid and specific	Standard methods, 1992; Hahma *et al.*, 2003; Fu *et al.*, 2005 ; Lien *et al.*, 2007.
RAPD, AFLP, PFGE, RFLP, AP-PCR, DNA fingerprint analyses	*E. coli, P.aeruginosa, Enterococci, Salmonella, H. pylori, enteric viruses, Cryptosporidium*	Tracing source of disease-causing infectious agents for distribution and prevalence	Grif *et al.*,1998; Sebat *et al.*, 2003; Purohit *et al.*,1996.
DNA Microarray technology	*E.coli, V. cholerae, Shigella, Salmonella,*	Efficient and accurate in detection of whole-genome expression and identification of multiplexed PCR products	Chizhikov *et al.*, 2001; Leonard *et al.*, 2003; Wolter *et al.*, 2008; Kim *et al.*, 2008; Li *et al.*,2008.
Gene probes	*E. coli*	Rapid differentiation of virulent strains from non-virulent	Betts,1999.
Bio-sensors	*E. coli, V. cholerae, Salmonella, Cryptosporidium, Giardia H. pylori*	Rapid and simple for culturable microorganisms Detection of bacteria bound on beads, membranes, fiber optics probe tips by laser excitation, acoustiogravimetric wave transduction or surface plasmon resonance	Osek, 2002; Rose *et al.*, 2007; Mutharasan, 2007; Zhua *et al.*, 2005.
Gene-chip Technology	*E. coli, Shigella, Salmonella,*	Specific, less expensive and sensitive to desired level of certain harmful microorganisms	Chizhikov *et al.*, 2001; Lipp *et al.*, 2003.
Solid-state Biochip	*H. pylori, Cryptosporidium, Giardia*	Rapid detection of a number of microbes and toxins without isolation and characterization of genetic elements in very short duration (minutes)	Hashsham, 2007.

microorganisms. Analysis of sequenced genomes of strains of *Escherichia coli and Helicobacter pylori* (Groisman *et al.,* 1993; Alm *et al.,*1999) indicates the differences in the nucleotide sequence in most of the same genes. Comparison of the differences in genomic sequences of pathogenic with non-pathogenic microorganisms forms the basis of different behaviour microorganisms and its isolates. Analysis of sequences which are absent in *Escherichia coli* led to the identification of pathogenicity island in *Salmonella typhimurium* (Karch & Bielaszewska, 2001). A complete sequence analysis of STEC 0157:H7 genome has been carried out by Karch and Bielaszewsk to understand the full spectrum of virulence characteristics and comparison of genetic evolutionary and phylogenetic relationship between pathogens. *Escherichia coli* K-12 sequence serves as the index genome, against which sequence of pathogenic enteric bacteria can be compared to determine and predict changes in gene pool and virulence traits in future.

Computation on integral component of genomic analysis has been used to determine the functions of genes based on homology sequence searching in different organisms, using a variety of comparison

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algorithms, such as, sequence comparisons, comparing genomic structure and modification searching (Groisman *et al.,* 1993). On the basis of the information obtained, inferences can be made about the evolutionary history and biosynthetic capacity of an organism, for the development of new antimicrobial agents. However, sequence homology searching has limitations of not detecting all genes with similar functions. In addition, bioinformatics provide computational tools needed for holistic biological research (www.infobiogen.fr/ services/ dbcat) by a) biological information infrastructure, which allows for interoperability among data resources and b) computational biology, i.e., the use of algorithmic tools to facilitate biological data analysis. This new technology will facilitate the study of all the genes of an organism in a systematic fashion by high throughput sequencing techniques paired with new algorithms for data analysis. Complementing the whole genomic approach to functional biology numerous web based information resources are available that provide databases and software tools for microbial data analysis to simulate the physiological behavior of microorganisms. A few examples are given in Box 1. The bioinformatics challenge will be to make such data accessible, comprehensible, and valuable to the research community.

Box 1. Web resources

GenBank	http://www.ncbi.nlm.nih.gov/
TIGR	http://www.tigr.org/tdb/mdb/mdbcomplete.html
IGRMD	http://www.tigr.org/tdb/mdb/mdb.html
AceDB	http://www.sanger.ac.uk/software/Acedb/
EcoGene	http://bmb.med.miami.edu/EcoGene/EcoWeb/
GOLD	http://igweb.integratedgenomics.com/GOLD/
EcoCyc	http://ecocyc.pangeasystems.com/ecocyc/
EMBL	http://www.ebi.ac.uk/embl.html/
GSDB	http://www.ncgr.org/research/sequence/
COG	http://www.ncbi.nlm.nih.gov/COG/
KEGG	http://www.genome.ad.jp/kegg/docs/intro.html,
WIT	http://www.cme.msu.edu/WIT/
SCPG	ftp://ftp.sanger.ac.uk/pub/pathogens
OUGC	http://www.genome.ou.edu/strep.html
MBGD	http://mbgd.genome.ad.jp/
	http://www.soi.city.ac.uk/~drg/research/ bioinformatics-list.html
BioCatalog	http://www.ebi.ac.uk/biocat/
Blast oftware	ftp://ncbi.nlm.nih.gov/blast/executables
	ftp://ncbi.nlm.nih.gov/blast/server
	ftp://ncbi.nlm.nih.gov/balst/netblast

Methodologies for detection

The occurrences of outbreaks of waterborne illnesses have attracted the attention of public health authorities as well as an interdisciplinary array of experts in fields of microbiology, engineering, epidemiology and risk assessment. The feasibility of detection and identification of microorganisms from water can be conceptualized into two groups based on the types of target or diagnostic molecules:

- phenotypic methods (based on protein, carbohydrate, lipid or other bio-molecules produced by target organisms)

Table 4. Selected water borne pathogens and techniques used for detection

Pathogen	Survival in water	Relative infective dose	Resist -ance to chlorine	Methods used for detection	Target gene
E. coli	High>1yr	high	low	PCR, Multiplex-PCR, RAPD-PCR, PFGE, RT-PCR, AFLP, Rep-PCR, Ribotyping	uidA, lacz, eaeA, hlyA, stx1, stx2, fliC,E-hly,LT1, ST1, STII, rfbE, tir
V. cholerae	Short	high	low	PCR, Multiplex-PCR, RT- PCR, Ribotyping	ctxA , uidR, rfb,toxR, tcpA, acfB, OmpU
P. aeruginosa	May multiply	high	moderate	PCR, Multiplex-PCR,RFLP, Rep-PCR, PFGE,RT-PCR, AFLP	VRE, toxA 16S, 23S Rrna, hbA, hybA
Enterococcus spp.	-	-	-	PCR, Rep-PCR, PFGE	efaA, esp, gelE, agg, cylMBA, VanA, VanB, VanC, ela
H. pylori	-	-	-	Rep-PCR,RAPD	23SrRNA
Salmonella sp.	moderate	high	low	RFLP,AFLP, RT-PCR, PCR, Rep-PCR, Multiplex-PCR, RT-PCR, PFGE, Ribotyping	VRE, ureC, urea,hpaA, VacA
Shigella sp.	Short	high	low	Multiplex-PCR nested-PCR, CFLP	ST14, ST11, uidA, rfbE
Enteric viruses	Long	low	moderate	PCR, RT-PCR, RFLP	uidR, uidA, lamb, SLT-I, SLT-II
E. histolytica	Moderate	low	high	PCR, nested-PCR	Beg9, VP-7 EH-1,ED-1, rRNA
Cryptosporidium	Moderate	low	high	RT-PCR, RFLP, RAPD, nested - PCR	COWP, 18SrDNA, TRAP-C, dhfr

(Ref: Bhanumathi et al., 2003 ; Haque et al., 2000; Heijnen & Medema, 2006; Fu et al., 2005; Mandrell & Wachtel, 1999; Eaton & Gasson, 2001; Hahma et al., 2003; Purohit et al., 1996; Corbella & Puyet, 2003; Geornaras et al., 1999; Malathum et al., 1998; Frahm & Obst, 2003; He et al., 2002; Johnson & Clabots, 2000; Belzer et al., 2009; Cebula et al., 1995; Dubois et al., 1997; More et al., 1994; Mirelman et.al., 1997; Queiroz et.al., 2001; APHA,1992; Kulkarni et.al., 1993; Fayer et.al., 2000)

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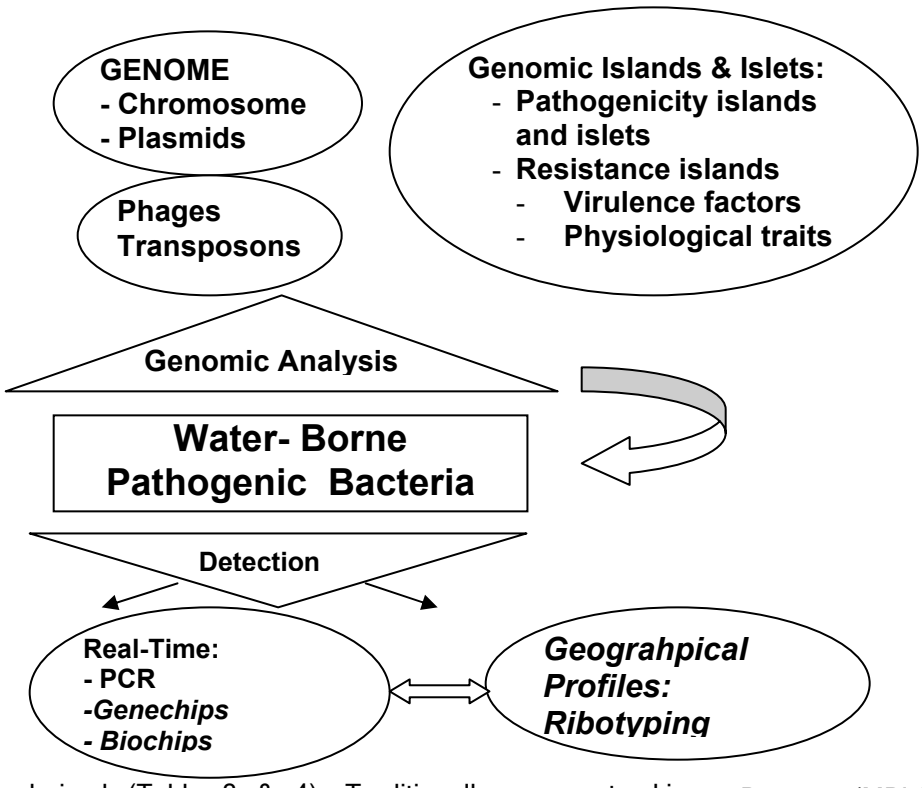
- genotypic methods (based on nucleic acids)

Therefore, various microbiological, biochemical and molecular methods have been proposed with the purpose of detecting subtle differences present within groups of microorganisms that can subsequently be used to identify the host or environment from which the organisms were derived (Table 3 & 4). Traditionally source tracking methods have targeted multiple antibiotic resistance (MAR) patterns, cell surface or flagellar antigens or biochemical tests designed to identify variation in the utilization of various substances that may be found within a particular host environment.

Fig. 5. Genomic analysis of flexible gene pool in water-borne pathogenic bacteria to aid geographical profiling and real-time detection



Numerous methods currently used show significant drawbacks including inadequate discrimination, limited availability of reagents, poor reproducibility and inability to quantitate the genetic relationships between isolates. Culture-based methods are time consuming, tedious, invariably nonspecific and multiple antibiotic resistance profiles have some inherent uncertainties as detection of one gene or specific enzyme product does not produce reliable identification (Table 3). While DNA based identification of microorganisms does not determine viability (Table 4) and no practical approach has been developed to specifically target majority of the uncultivable species in the environment. Thus new approaches are needed for development of full range of pathogen detection techniques that will lead to a strong, integrated detection system.

An international expert meeting in Interlaken concluded (Karch & Bielaszewska, 2001) that the application of molecular methods has to be considered in a framework of a quality management for drinking water (Fig. 5). In last decade, the development and extensive use of high resolution molecular typing systems based on direct analysis of genomic polymorphism have greatly improved the understanding of epidemiology of infectious diseases (Rose & Grimes, 2001). Methods that index chromosomal DNA polymorphism are the best options for comparative typing. Epidemiologic typing can be used to confirm and delineate the patterns of transmission, evaluation of control measures by documentation of prevalence time and reservoirs of epidemic organisms.

Real-time polymerase chain reaction

Recent advances in PCR technology have facilitated the development of real-time PCR with greatly reduced amplification period and improved method for detection of amplified target sequences. In real-time PCR, the target gene is amplified and simultaneously recognized and monitored by the fluorescent probe moiety. As the reaction in this method is homogenous the risks of cross-contamination are minimized and downstream analyses are limited. Recent developments of fluorogenic nucleic acid probes such as Molecular Beacons (MB's) confer new dimension to PCR, for which results have become quantitative and available in a real time manner, for ecological and epidemiological studies to determine what species are present in the population. MB's are oligonucleotides probes that possess differential fluorescent properties based on the relative stability between its duplex forms (hairpin and the probe target hybrid). The advantage of MB's is their extraordinary specificity, as no increase in fluorescence is observed even in the presence of a target strand containing a single nucleotide mismatch (Kim *et al.,* 2008; Li *et al.,* 2008).

Other specific fluorescent oligonucleotide probes for real time monitoring of polymerase chain reaction are TaqMan, Minor Groove Binder (MGB), Fluorescence Resonance Energy Transfer (FRET) and Scorpion. Highly reliable, real-time PCR has the advantages of a regular PCR in addition to the demonstration of a supplementary level of specificity, allele discrimination (Tyagi *et al.*,1998; Lee *et al.*, 2009). Reischl *et al.* (2002) reports amplification and detection of *eae, hlyA, stx1* and *stx2* in *Escherichia coli* using real-time PCR. Multiplex PCR with

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molecular beacons has been used for detection of pathogenic retroviruses in human blood samples and Shiga-toxin producing bacteria in feces (Vet *et al.*,1999; Belanger *et al.,* 2002). The speed of detection and the availability of potential subtyping information make RT-PCR a better alternative to block cyclers PCR assays. Quantitative detection of multiple target organisms within a single sample, preferably in real time (Nathalie *et al.,*2001; Mackay, 2007) could be an ideal system in monitoring drinking water contamination.

Ribotyping

Phylogenetic analysis is necessary for emerging pathogens with partially known virulence factors. Ribotyping, a versatile and widely used strategy of southern blot analysis of bacterial genome polymorphism, has emerged as one the most powerful tools and 'Gold Standard' of molecular epidemiology (Grimont & Grimont,1986; Henegariu *et al.,*1997). Ribotyping uses restriction fragments of ribosomal RNA genes for characterization of organisms (Amann & Ludwig, 1994, 2000). It is a robust method with excellent reproducibility and stability during the course of outbreaks and is commercially available in a fully automated and well-standardized format. Grif *et al.* (1998) used *EcoR*I as restriction enzyme for discriminating epidemiologically related and unrelated *Escherichia coli* 0157:H7 isolates. Ribotype pattens of many pathogens such as *Enterobacteriaceae, Pseudomonas* sp., *Helicobacter alvei* have been produced. Ribotyping is being increasingly explored for differentiating fecal *Escherichia coli* of human origin from pooled fecal *Escherichia coli* isolated of non-human origin (Parveen *et al.,* 1999; Carson *et al.,* 2001). Ribotyping can generate an electronic riboprint database for rapidly investigating emergence of virulent strains provided precise geographic, socio-economic and medical data is available.

Oligonucleotide microchips

Oligonucleotide microchips are used in determinative and environmental microbiology and provide powerful format for the systematic exploration of natural microbial diversity. In addition to their use in phylogenetic group identification, microchip array can be used to evaluate sequence motifs that have yet to be identified in a habitat with microbial populations of interest. The use of microchips for determinative studies provides several advantages over conventional hybridization formats. Hundreds of different oligonucleotides can be immobilized on a single microchip allowing simultaneous detection of a great variety of different microorganisms in a single sample and can be used 20-30 times without noticeable deterioration of the hybridization signal. It can be used for direct analysis of environmental populations thus, not requiring prior amplification of the target nucleic acids. Oligonucleotide microchips have been used to discriminate *Escherichia coli, Salmonella* and *Shigella* sp. (Chizhikov *et al.,* 2001). In addition, direct detection of source-specific genotypic markers in water samples without isolation holds promises. However, for its wider applicability, the possible temporal diversity, geographical limitations and survival of these source-specific genotypic markers in the environment need to be assessed. These microchips technologies are promising for meeting the future demands of biochemical analysis (Table 3).

Conclusion

Little is known about the contribution of chromosomal evolution and genotypic variation in pathogen population that poses a major barrier to disease control. The availability of the complete genomic sequence of a variety of microorganisms, coupled with new technologies for large-scale analysis of gene expression and function, has provided an abundance of new opportunities for understanding complex biological properties of microbial pathogens. Sequence information along with analysis of the sequenced data is opening up new horizons for studying microbial evolution and pathogenesis as well as development of new diagnostic and monitoring techniques. Future developments in signal detection and miniaturization technologies will provide real-time monitoring and diagnostics for rapid assessment of microbial pathogens. Establishment of such highly parallel and specific methods is essential to reduce the health risk from microbial pathogens present in water source. Highly effective and real-time diagnostic systems with a wider coverage of microbial pathogens therefore, play an integral role in facilitating an effective response to be provided against water borne infections.

But to be more useful and usable, the different technologies for assaying microbial water quality must be cost effective fulfilling the needs of public health and environmental regulators as most of the developing countries still do not have the necessary sophisticated laboratory infrastructure to comply with safe drinking water requirements. It is strongly felt that development of a database system at national level on real-time monitoring and diagnostics systems developed at various national laboratories/institutes is essential so as to ease the access of the modern technologies as and when required. It is equally important to create awareness among public, medical practitioners and the entrepreneurs on the available technologies.

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