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The Need and New Tools for Surveillance of *Escherichia coli* Pathogens

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Summary

Among foodborne pathogens, diarrhoeagenic *Escherichia coli* is of major concern because of its commensal status, abundance in the natural environment, and ability to acquire virulence determinants by horizontal gene transfer from other microbes. From enterotoxigenic *E. coli* (ETEC) strains to the more virulent enterohemorrhagic *E. coli* (EHEC), the mechanisms of pathogenicity within this species are intriguing. Recent advances in molecular diagnostics are providing novel tools for improved rapid detection and quantification of this and other pathogenic bacteria from clinical, food, and environmental specimens. These include simple and inexpensive colorimetric and immunological methods to more elaborate nucleic acid-based assays that combine extreme specificity to unparalleled sensitivity and high sample throughput. This review summarizes the current state of *E. coli* pathogenesis with emphasis on the need for incorporating detection and surveillance tools as part of pre- and post-harvest food safety ideals.

Key words: Escherichia coli, diarrhoea, molecular pathogenesis, toxins, antigens, antibodies, diagnostic targets, surveillance

Introduction

Diarrhoea is a major global foodborne disease caused by a wide variety of microorganisms ranging from viruses to parasites. However, it was not until 1982 when a major outbreak of *E. coli* O157:H7 swept through the United States, which was traced to contaminated hamburgers sold at a popular chain of restaurants, that people started considering *Escherichia coli* as a serious foodborne pathogen (*1*). Otherwise, *E. coli* is generally regarded as harmless commensal bacterium inhabiting human and animal intestines, producing vitamins and enhancing vertebrate innate immunity. Occasionally, common *E. coli* strains are associated with opportunistic infections such as septicemia (*2,3*). In fact, certain strains of this bacterium can cause serious infections that are often fatal, particularly in infants and children. According to a WHO report (*4*), approximately 11 million children under the age of five die because of *E. coli*-mediated gastroenteritis, 4 million of them in the first two months of life. Such an abundant microorganism associated with human beings and other animals (especially ones that share the same niche) and the ease with which it may be spread through food, obligates healthcare providers, foodservice personnel, and many others in the public sector to maintain heightened vigilance and constant surveillance (*5,6*). This review is meant to highlight this awareness and bring to light some of the latest developments that could help in tracing and monitoring pathogenic forms of *E. coli* in food and clinical samples.

E. coli Pathogens – Virulence Profile

E. coli is a straight, rod-shaped, Gram-negative bacterium from the family *Enterobacteriaceae*. Some strains of *E. coli*, during the course of evolution, have acquired virulence factors and mechanisms that aid them in invading the host (*7*). These harmful varieties cause serious

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intestinal and urinary tract infections. Such pathogens are categorized into 5 major types based on their virulence mechanisms (*5*): (*i*) enterotoxigenic *E. coli* (ETEC), (*ii*) enteropathogenic *E. coli* (EPEC), (*iii*) enterohemorrhagic *E. coli* (EHEC), (*iv*) enteroinvasive *E. coli* (EIEC), (*v*) enteroadherent *E. coli* (EAEC) or enteroaggregative *E. coli* (EAggEC).

All of these cause diarrhoea and other complications that are specific to each pathotype (*8*). ETEC causes the least complicated of the diseases by producing a cholera-like labile toxin (LT) or heat-stable peptide toxin. The diarrhoea caused by ETEC is watery and leads to severe dehydration if the ion and water losses are not replenished. Foreign travellers visiting developing countries often suffer this self-limiting diarrhoea, commonly referred to as traveller's diarrhoea (*8*). In contrast, EPEC causes persistent diarrhoea in infants and children under two years of age, accounting for higher infantile morbidity and mortality, particularly in developing countries such as India. Diarrhoea due to EPEC lasts for more than 10 to 12 days and the toxicoinfection causes extensive destruction of the intestinal brush border membrane, leaving the affected children severely malnourished (*9*). EHEC often causes bloody diarrhoea (hemorrhagic colitis, sometimes referred to as HC) and in 10 % of the cases could lead to haemolytic uremic syndrome (HUS) (*10*), in which the red blood cells are destroyed and the kidneys fail. EIEC also elicits HC, clinically indistinguishable from shigellosis or bacillary dysentery. Unlike other *E. coli* pathogens, EIEC invades colonic epithelium using plasmid-encoded invasion proteins to colonize and spread subepithelially. This causes widespread cell destruction, severe inflammation and ulceration of the tissue contributing to higher mortality and morbidity among children (*11*). Enteroaggregative *E. coli* (EAEC or EAggEC) may cause chronic diarrhoea in children and immunocompromised persons (*12*). Recently, the emergence of Shiga toxin-producing *E. coli* (STEC), a subclass of EHEC, has been reported from more than 30 countries including India (*13*). Uropathogenic *E. coli* (UPEC) are the most common cause of urinary tract infections (UTI) and may lead to bacteriuria, cystitis and pyelonephritis (*12*). These strains affect women more commonly than men, although none are considered foodborne pathogens and therefore will not be discussed further.

Of late, there has been tremendous interest and serious investigations in elucidating how *E. coli* could have evolved into a major pathogenic class. This effort has resulted in many interesting observations regarding intricate host-pathogen interactions. Particularly for *E. coli*, the infection strategies of its pathogenic forms have both common themes and characteristically distinct steps specific to each type (Table 1, *8*). The major outline of the underlying pathogenic mechanism is that, following the ingestion of contaminated food or water, these bacteria first colonize the mucosal layer of intestinal epithelium. Evasion of the hosts' innate immune response either by limiting infection to the epithelial layer, as in the case of ETEC and EPEC, or by remaining within epithelial cells, as in the case of EIEC, allows them to multiply and cause damage to the host. The differences among the different pathotypes are based on how each causes damage to the host, whether the strain is invasive or not, and whether they secrete toxins or not. Among the different *E. coli* pathogens – ETEC, EPEC, EHEC, EIEC and EAggEC share many common features as they all colonize (albeit briefly) intestinal tissue and cause diarrhoea (Table 1, *14*). Unravelling the characteristic mechanistic details at the molecular level has resulted in identifying targets for diagnostics, as early detection and constant surveillance appears to be the best way to minimize complications and arrest the spread of the pathogen.

Enterotoxigenic E. coli

Enterotoxigenic *E. coli* (ETEC) is among the most common *E. coli* diarrhoeal pathogens. In the third world countries about 0.7 million childhood deaths due to ETEC diarrhoea are reported every year (*15,16*). ETEC produces two types of toxins – the heat-labile toxin (LT)

Table 1. Clinical, pathological and epidemiological characteristics of diseases and the major virulence-associated determinants of the five principal pathotypes of diarrhoeagenic *Escherichia coli*

Pathotypes	Clinical presentation	Intestinal pathology	Susceptibility	Adhesions/ Invasions	Secreted toxins
EPEC	Non-specific gastroenteritis	Attaching-effacing lesions throughout the small intestine	Children under 2 years of age in developing countries	BFP, intimin	*Esc*
ETEC	Watery, cholera-like diarrhoea	Attaches to small intestinal mucosa through colonization factor antigens (CFA)	Children in developing countries; travellers to those countries	Colonisation factor antigens *e.g.* CFAI/CFAII	LT and ST
EIEC	Bacillary dysentery	Inflammation and disruption of the mucosal membrane, mostly of the large intestine	All ages; more common in developing countries	Invasion-plasmid antigens (Ipa)	*Shigella* enterotoxin (Sen)
EHEC	Bloody diarrhoea	'Hemorrhagic colitis', attaching-effacing lesions confined to the large intestine; necrosis in severe cases	Children and the elderly in industrialized countries	Intimin	Verotoxin (Vt), Shiga toxin (Stx)
EAEC	Persistent diarrhoea	Biofilm formation, inflammation; hemorrhagic necrosis of villi	Children in developing countries; travellers to those countries	Aggregative adherence fimbriae (AAF)	EAST; Pet and ShET-1

and the heat-stable toxin (ST) (*17*). These are shown to be delivered *via* their outer membrane vesicles (*18*). LT is an A-B type of toxin, exhibiting 80 % homology with cholera toxin (*19*). As in cholera pathogenesis, the catalytic A subunit is delivered into host cells through a channel formed by five B subunits bound to the gangliosides present on the intestinal epithelial membrane (*19*). Once inside the intestinal epithelium, the A subunit splits into fragments A1 and A2, the former of which catalyses the transfer of the ADP-ribosyl group from NAD to a critical Arg_{201} of a G-protein. Normally, in its GTP-bound state, this protein activates adenylate cyclase to produce cAMP in a regulated manner. ADP ribosylation prevents conversion of GTP to GDP, and thereby adenylate cyclase is continuously activated to produce cAMP (Fig. 1, *20*). The constant production of cAMP, in turn, activates cAMP-dependent protein kinase A (PKA), eliciting a cascade of reactions that ultimately phosphorylate various ion channels. Phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) activates the chloride channel to release chloride ions from the host intestinal crypt cells. Absorption of Na^+ and Cl^- by the surface (villi) cells is decreased, resulting in perpetual loss of ions and water (*21*).

The heat-stable ST is a cysteine-rich small peptide of 18–48 amino acids. Based on the activity and the number of amino acids, ST is divided into STa (ST-I) and STb (ST-II) (*22*). The smaller peptide consists of 18–19 amino acids with six cysteine residues forming a cage made of three disulphide bridges that directly activates membrane-bound guanylate cyclase on the epithelial membrane by mimicking the natural intestinal hormone guanylin. Continuous production of cGMP then brings about activation of membrane channels to release ions and water (*23*). STb forms two disulphide bonds with 48 amino acids and helps in the release of serotonin, prostaglandin E2 and Ca^{2+} (*22,24*). Apart from LT- or ST-producing ETEC, many other ETEC strains secrete both toxins. One of the salient features of ETEC pathogenesis is the ability to produce multiple fimbriae in order to attach to small intestinal mucosa using specific receptors like colonization factor antigens CFAI, CFAII, PCFO159 and PCFO166 for effective colonization (*25,26*). This zoonotic pathogen has these colonizing factors and toxin-coding genes in the plasmid and therefore the risk of horizontal gene transfer generating new variants could be high, especially under the conditions of co-inhabitation of animals and humans. It is a common observation that many virulence factors like cytotoxins and colonizing factors are coded by mobile elements and acquired by pathogens by horizontal gene transfer; a recent mathematical model has been proposed that directly relates mobile virulence determinants and social evolution in pathogenic bacteria (*27*).

Enteropathogenic E. coli

Enteropathogenic *E. coli* (EPEC) was the first among *E. coli* strains to be identified as a diarrhoeal pathogen from outbreaks in neonatal and pediatric wards of hospitals in the UK and other European countries (*28,29*). EPEC is more severe in infants under six months of age (*4*), and in developing countries accounts for 30 % mortality in children under the age of five (*30,31*). Originally identified as the etiological agent of infant deaths in pediatric nurseries in England and many other developed countries, EPEC has many intricate adaptations to persist in the intestine and cause protracted diarrhoea (*14*). Though the high profile EHEC shows similar histopathology, it is a variant of typical EPEC lacking bundle-forming pili but additionally secreting Shiga toxin, which inhibits protein synthesis and contributes to tissue damage. There are other molecular variations, such as in the sequence of the C-terminal portion of a major virulence factor (intimin) involved in intimate attachment to host tissue (*32*). EPEC does not penetrate the epithelium but remains in intimate contact with the surface of M cells and host enterocytes, leading to histopathological changes termed attaching and effacing (A/E) lesions (*32*). In terms of understanding host-pathogen interactions, this is one of the best-studied models. As depicted in Fig. 2, the three stages of this toxicoinfection are: (*i*) localized adherence to intestinal epithelial cells, (*ii*) signal transduction and host cytoskeletal rearrangement, (*iii*) intimate attachment to epithelium causing destruction of microvilli (A/E lesions) and diarrhoea (*33*). Upon con-

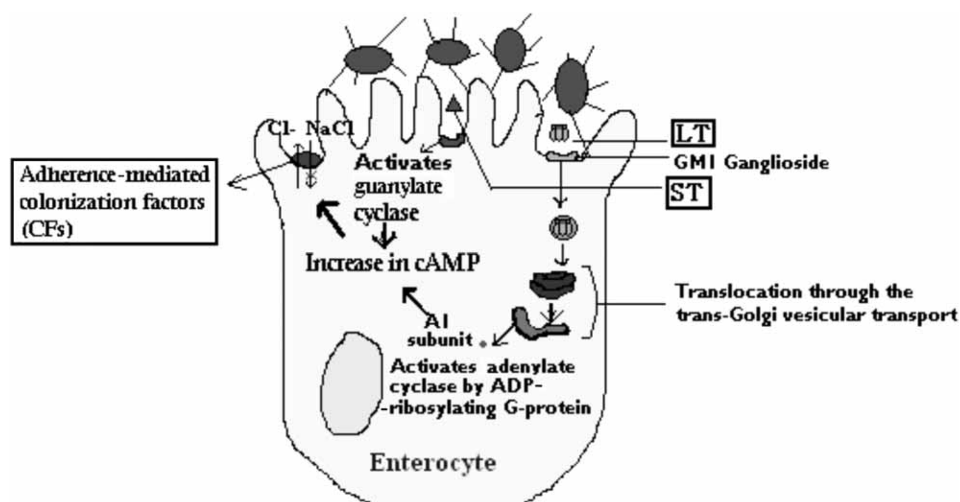

Fig. 1. ETEC pathogenesis and targets of diagnosis

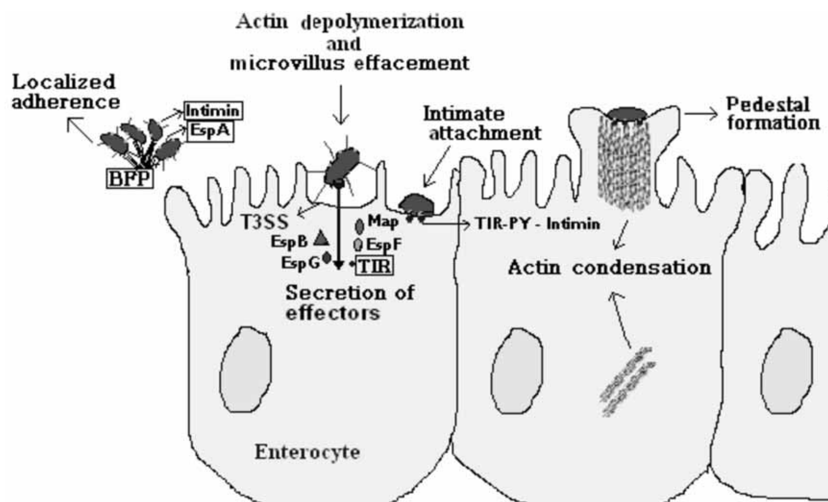

Fig. 2. EPEC pathogenesis and targets of diagnosis

tact with the host, this as well as many other Gram-negative bacterial pathogens (including EHEC and EIEC) express special secretory machinery termed type III secretion system (T3SS) (*34*). This well-characterized system delivers virulence factors expressed in the bacterial cytoplasm into the host cell through a protein tubule inserted into the host cell membrane. In EPEC, the T3SS subunits, the effector proteins EspA, EspB, EspD, EspF, and the translocated intimin receptor (Tir) are coded by a 35-kb long pathogenicity island present on the nucleoid. This region is called locus of enterocyte effacement (LEE), named after the type of damage it causes (*35*). The biochemical nature and pathogenic role of these effector proteins have been studied intensely (*36*).

EspA forms the transmembrane structure that facilitates toxin delivery. EspD plays a secondary role in the formation of EspA-associated appendages and the formation of pores in the host membrane (*37*). Both EspB and Tir have been found in host cytoplasmic and membrane fractions, although the role of EspB in the host-pathogen interaction is not yet clearly understood (*38–40*). Tir is an EPEC receptor expressed on the host cell surface after getting phosphorylated in the host cell cytoplasm (*41*). It binds to a 90-kDa outer membrane protein of EPEC termed intimin (*42*). For many years, intimin had been considered an attractive diagnostic and prophylactic target, but soon many intimin variants were identified, revealing polymorphism as an EPEC survival strategy (*43,44*). Bundle-forming pili (BFP) mediate the characteristic localized microcolony formation of EPEC in *in vitro* cell culture assays. However, many atypical EPEC isolates have been described recently, indicating that this pathogen has tremendous potential to exist as minor variants and persist in human and animal populations. Recently we have demonstrated the cytopathic effects of cell-free outer membrane preparations of EPEC and constitutive expression of maltoporin in a virulence-associated manner (*45,46*), indicating a wide ranging host-pathogen interaction involving many outer membrane proteins (*47*). A plasmid encodes what is referred to as *E. coli* adherence factor (EAF); this virulence plasmid is 55–70 MDa in size, and encodes BFP and proteins that regulate LEE (*48*). In short, this zoonotic pathogen with human and animal specific pathovars has the potential to emerge as a major threat by acquiring new traits *via* horizontal gene transfer.

Enterohemorrhagic E. coli

In 1999, Centers for Disease Control and Prevention's (CDC's) Foodborne Diseases Active Surveillance Network (FoodNet) estimated about 76 million cases and 5000 deaths due to foodborne illnesses. Enterohemorrhagic *E. coli* (EHEC) alone contributes to 73 000 cases and 60 deaths every year. But, according to the report released in 2005, FoodNet revealed that the incidences reduced to half when compared to the statistics of 1999 (*49,50*). Annually, a loss of between $6.5 and 34.9 billion has been attributed to foodborne illnesses in the USA alone (*51*).

EHEC, often referred to as *E. coli* O157:H7 based on the O and H antigenic serotypes, shot into prominence in 1982 when it was identified as a dangerous foodborne pathogen responsible for a hemorrhagic colitis outbreak after consumption of contaminated hamburgers from a fast food restaurant in the USA (*52,53*). Since then, EHEC has been associated with foodborne outbreaks in developed industrialized countries worldwide, causing about 250 deaths every year (*54,55*). Cattle are the only proven reservoir of EHEC, accounting for its spread through raw or undercooked ground meat products and raw milk. However, it has been shown that EHEC also spreads through the consumption of contaminated fruit, vegetables, water, apple cider, and other minimally processed foods, which makes it one of the most significant foodborne pathogens worthy of constant surveillance (*4*).

EHEC is a rather robust pathogen. It can survive extreme temperatures from 7 to 50 °C, with an optimum temperature of 37 °C. It has been found to grow in acidic foods at a pH=4.4 and in foods with a minimum water activity (a_w) of 0.95. It can be destroyed by thorough cooking of foods until an internal temperature of 70 °C (155 °F) or higher has been reached (*56*).

Signs and symptoms due to EHEC consist of abdominal cramps and initial watery diarrhoea, followed by bloody diarrhoea with little or no fever. The severe gas-

trointestinal disease is accompanied by haemolytic uremic syndrome (HUS) in about 10 % of the cases (*57,58*). Approximately 8 % of the affected individuals experience neurological abnormalities such as seizures, cortical blindness, deposition of platelets and fibrin with the glomeruli, with coma (*59*) and death occurring in 3–5 % of the cases (*60*). The most common EHEC serotype responsible for the outbreaks is O157:H7. Other serotypes including O5, O26, O91, O111 and O113 have also been recovered from patients (*61*).

As mentioned above, EHEC, the pathogen that actually turned the spotlight on *E. coli* as an emerging threat, is essentially derived from EPEC (except for lacking bundle-forming pili) (*5,8,62,63*). As with EPEC, the major virulence factors (Fig. 3), causing A/E lesions, are encoded by a 35.6-kb locus of enterocyte effacement (LEE) (*64*). The 41 open reading frames (ORFs) of LEE are organized into 5 major operons. Though the actual mechanisms are not clearly understood, LEE also plays a vital role in regulating virulence genes depending on environmental factors such as temperature, osmolarity and quorum sensing (*65*). A distinguishing factor in EHEC pathogenesis is the bacteriophage-encoded Shiga toxin (Stx) primarily responsible for pathogenesis (*66,67*).

Stx is a protein synthesis inhibitor that targets renal arteriolar, aortic, brain endothelial, proximal and distal renal tubular epithelial, and glomerular endothelial cells, monocytes and lung epithelial cells (*68*). Stx acts directly on human proximal tubular cells *in vitro* and is the major virulence determinant in HUS. The distal renal tubular cells, on the other hand, express glycosphingolipid globotriaosylceramide (Galα1–4Galβ1–4glucosyl ceramide, Gb3) and undergo apoptosis after binding Stx (*69,70*). When the bacteria colonize the intestine, translocation of Stx1 and Stx2 across the intestinal epithelium takes place. Both Stx1 and Stx2 have A and B subunits; the catalytic A subunit is transported into the cells through a membrane-bound complex of five B subunits. Within the infected cell, the *N*-glycanase activity of the A subunit eliminates a specific adenine base from the 28S rRNA of the 60S ribosomal subunit. Since this adenine base is on a loop of rRNA that is important for binding of elongation factor, the toxin is able to shut down protein synthesis in a targeted cell. In the case of the glomerular endothelium, the cells become swollen and detach from the underlying basement membrane. The damage varies from mild cellular edema to severe mesangiolysis, which leads to lethal toxic injury. While five variants of B subunit preferentially bind to the globoceramide Gb3, Stx2e binds preferentially to globotetraosylceramide (GalNAcβ1-3Galα1-4Galβ1-4glucosyl ceramide). These different binding properties provide the toxin with the ability to target different cells (*71–73*).

The only effective method of controlling EHEC outbreak is heating food material to an internal temperature of 70 °C (cooking thoroughly or pasteurization) or irradiation of sprouted seeds, milk *etc.* to kill the bacteria. Examination of reservoir animals before slaughter, and educating abattoir workers that the control of hygiene and cross-contamination risks could minimize the possible introduction of pathogens in the slaughtering environment. Similarly, educating farm workers in principles of good hygienic practice should be carried out in order to minimize the contamination.

Enteroinvasive E. coli

Enteroinvasive *E. coli* (EIEC) strains are biochemically, genetically and pathogenically very similar to *Shigella*. EIEC is nonmotile and negative for lactose fermentation and lysine decarboxylase tests (*74*). EIEC causes bloody diarrhoea that is clinically indistinguishable from that caused by *Shigella* (*5*). The expression of virulence genes is transcriptionally regulated by temperature; bacteria which are invasive when grown at 37 °C become non-invasive when grown at 30 °C (*75,76*).

Both of these pathogens, EIEC and *Shigella*, infect the large intestinal epithelium from the basolateral side. In the intestinal tract, amidst differentiated epithelial cells, undifferentiated patches of M cells (microfold cells) are present for luminal content sampling and presenting to underlying macrophages (Fig. 4). Once inside the macrophage, the bacteria escape the phagosome and trigger apoptosis of the macrophage. EIEC then get released at the basal side of the host large intestinal epithelium. Here the pathogen makes use of its T3SS to translocate

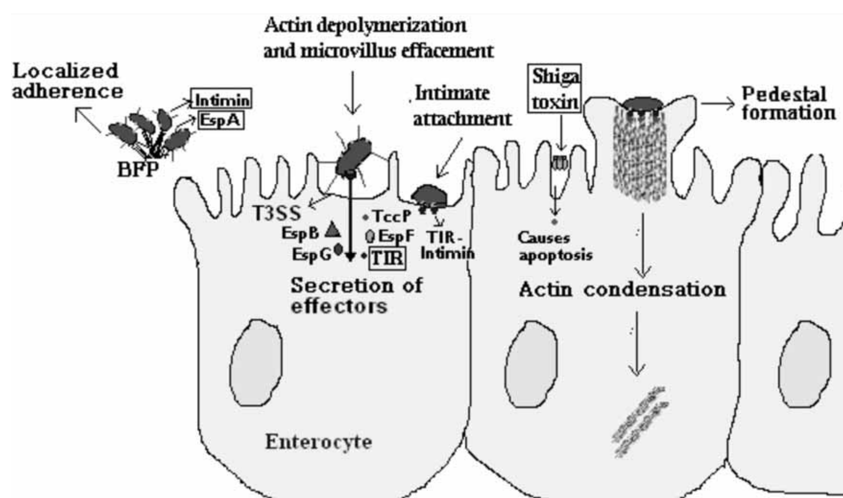

Fig. 3. EHEC pathogenesis and targets of diagnosis

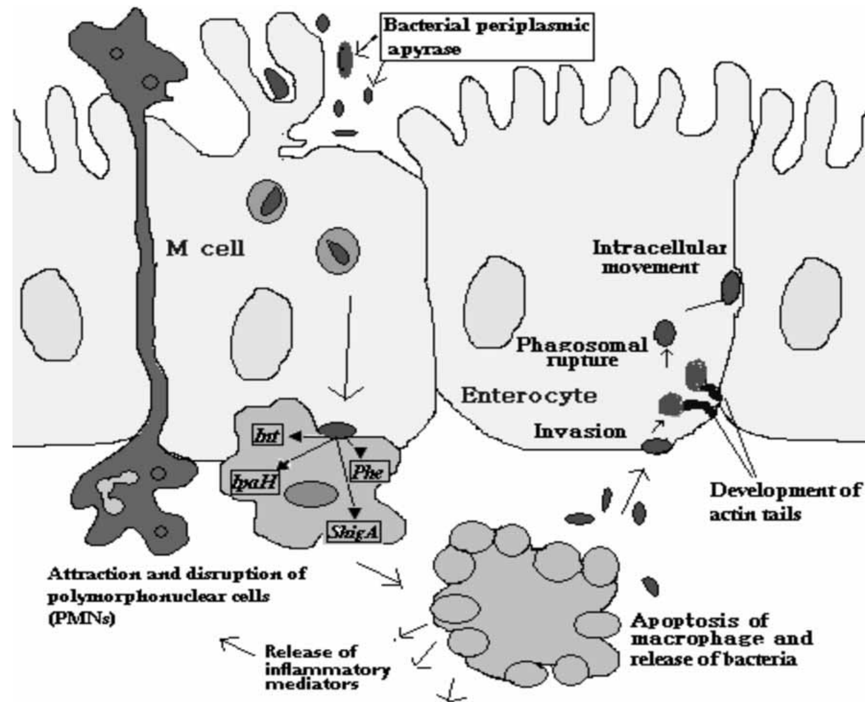

Fig. 4. EIEC pathogenesis and targets of diagnosis

the effector proteins (a group of invasion proteins) into epithelial cells, which causes host cytoskeletal rearrangement and induces micropinocytosis to facilitate the invasion from the basolateral side. Meanwhile, these events trigger the host inflammatory response, attracting polymorphonuclear cells that migrate to the luminal side, destroying tight junctions between the epithelial cells. The pathogen takes advantage of the breach in the epithelial cell tight junctions and infiltrates the basal side of the epithelium.

Once inside the epithelial layer, EIEC accomplishes two important tasks. First, the bacteria escape the inflammatory reaction triggered earlier, causing the host cells to suffer the damage due to these innate reactions. Secondly, the pathogen induces actin tail formation, and thus uses the host cytoskeletal filaments for mobilization and dissemination. This subepithelial colonization can be overpowered only with a full-fledged humoral immune response, which results in tissue destruction diagnosable as blood and mucus in the stool (*77*). It should be obvious from the above description that a large number of virulence factors have to take part in a concerted manner to cause the symptoms of bloody diarrhoea. Unlike EPEC and EHEC, most of the virulence factors (T3SS, invasion plasmid antigens (Ipa) and secretory plasmid antigens) are encoded by 140-MDa plasmid called the invasion plasmid (Fig. 4, *78*).

EIEC are very successful epidemic pathogens in developing countries, contributing to increased mortality and morbidity rates among children. In fact, attempts to develop vaccines based on such sound molecular pathogenesis knowledge still have not been a commercial success. Prevention, early detection, and control with appropriate antibiotics seem to be the best way to reduce morbidity and mortality.

Enteroaggregative or enteroadherent E. coli

This is a recently recognized *E. coli* pathogen and is less well studied than the other pathotypes. Originally, Cravioto *et al.* (*79*) exploited adherence patterns of EPEC using *in vitro* cell culture assays to distinguish between various pathogenic *E. coli* strains. Accordingly, this group classified *E. coli* pathogens as diffusely adherent *E. coli* (DAEC) and localized adherent *E. coli* (LAEC); the latter was redefined as EPEC (*79,80*). However, such a procedure led to the discovery of enteroaggregative or enteroadherent *E. coli* (EAggEC or EAEC), which in rabbit ileal loop experiments showed the adherent bacteria embedded within a mucosal biofilm, causing shortening and hemorrheagic necrosis of villi, mucoid stools and persistent diarrhoea (*81,82*). The biofilm formation and characteristic brick-like arrangement during colonization have been well studied. Bacterial adherence has been shown to be mediated by bundle-forming EAEC fimbriae (also called aggregative adherence fimbriae or AAF/I) (*83*). Expression of a 38-amino acid-long heat-stable enterotoxin (EAST-1), identified by Savarino *et al.* (*84*), has been shown to be a characteristic feature of EAEC. Eslava *et al.* (*85*) identified a 108-kDa plasmid-encoded toxin (Pet) that induces exfoliation of rat intestinal enterocytes. Thus, the pathogenesis of EAEC has three distinct stages (Fig. 5). First, the bacteria adhere to the host cells through AAF. Secondly, the bacterium produces mucus in large amounts leading to biofilm formation, which helps in colonization under protected cover. Finally EAEC secrete toxins and inflammatory mediators that damage intestinal mucosa (*12*). EAEC is being increasingly isolated from diarrhoeal specimens and from the available clinical statistics it appears to be a significant pathogen (*86*).

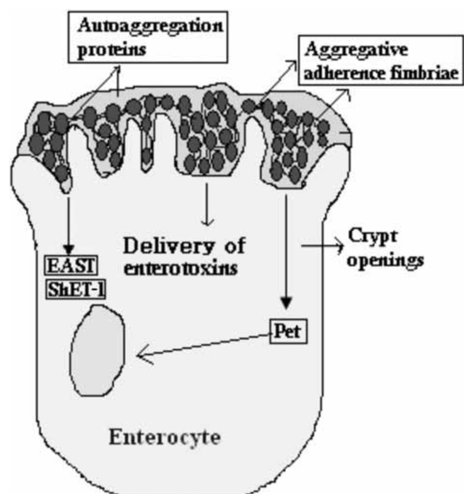

Fig. 5. EAEC pathogenesis and targets of diagnosis

Need for Surveillance

E. coli-mediated illnesses are normally food- and waterborne. Consumption of uncooked vegetables (or many 'ready-to-eat' fresh foods), ground beef, unpasteurised milk, juice and drinking water, or handling of food by affected person(s) are the main causes. Organically grown vegetables and fruits pose a risk in this regard, as they are essentially grown on animal manure. While people of all age groups may succumb to *E. coli* toxicoinfection, young children, the elderly, and those with compromised immune systems such as AIDS patients are the most severely affected (*5*). Contamination by *E. coli* is generally not apparent, as it does not alter sensory qualities of affected foods (*5,8*). Furthermore, *E. coli* pathogens such as O157:H7 in hamburgers often survive warming or mild baking (such as reheating leftover foods). In many cooking procedures currently in practice, manual handling of food ingredients (such as kneading and mixing) cannot be avoided. Therefore, it is important to make sure that the final preparation is free of contamination, especially if the food is intended for public consumption, by employing appropriate detection procedures and following strict hazard analysis and critical control points (HACCP) at all stages of food processing.

E. coli Prevalence in Communities

Globalization of the food supply, especially from developing to developed countries, has accentuated the problem of disease spread. This is best exemplified in the case of the recent bird flu scare. In a recent unpublished study conducted by our lab on 64 *E. coli* strains identified from diarrhoeal stool samples of 203 hospitalized infants under two years of age suffering from persistent diarrhoea for more than a week, we have identified 24 EPEC and six EHEC strains, but no ETEC or EIEC ones. However, the nature of the remaining 34 strains has to be established by biochemical and genetic characterization. These *E. coli* strains are not nosocomial but actually acquired from impoverished communities in India. Since many foods and food additives are derived from such regions, it is imperative that commercial food preparations are tested for *E. coli* pathogens and subjected to surveillance.

Antibiotic Resistance among *E. coli* Pathogens

Not only are *E. coli* pathotypes collectively adept at acquiring virulence determinants, but diarrhoeagenic *E. coli* also acquire multi-drug resistance to common antibiotics and are emerging as a major health threat to children with long lasting consequences that could manifest into socioeconomic problems later (*87*). *E. coli* pathogens possess a remarkable array of mechanisms to overcome the effects of antimicrobial agents (*88*). Acquisition of resistance genes by horizontal gene transfer mediated by plasmids and transposons is currently thought to be significant in the development of this multi-drug resistance (*89*). Such resistance determinants are most probably acquired by pathogenic bacteria from a pool of resistance genes in other microbial genera, including antibiotic--producing organisms, as well as phage (*90–94*). In our study, we have found that most clinical *E. coli* strains in our collection are resistant to most of the commonly prescribed broad-spectrum antibiotics. This underscores the importance of detecting drug resistance genes in these pathogens during surveillance.

Tools for Early Detection and Surveillance

All overseas validations of diagnostic products are generally monitored by the Association of Official Analytical Chemists (AOAC) instead of FDA and USDA. AOAC recommends validation of acidity levels, fat and moisture content, bacterial competition, and salt concentrations for food products (*95*). The existence of pathogenic forms of *E. coli* has been known for nearly five decades and many methods have been developed for their identification. Among early approaches (and even today in many instances), serotyping is the most common process (*5,8*) because it is the most accepted means of detecting pathotypes associated with disease (Fig. 6). However, this simplistic scenario is getting complicated as more and more serotypes are brought to the notice of clinicians. Presently, approximately 173 O antigens, 103 K antigens, and 56 H antigens have been identified and no doubt further types will be added in the future. Some serotypes of *E. coli* are included as EPEC and some of the serotypes are common to different pathotypes. For example, serotypes O126 and O127 are common to both EPEC and EAEC (*86*), whereas H2 and H6 are found in EPEC and EHEC. Therefore, as an alternative to serotyping, it has become necessary to identify highly conserved characteristic protein targets among pathotypes. A few of these targets have already been identified and exploited based on detailed molecular pathogenesis studies.

Furthermore, although many techniques with high specificity and sensitivity are useful in research laboratories, techniques such as polymerase chain reaction (PCR) are not necessarily suitable for peripheral labs or in field study. Therefore, the need of high priority is to develop simple and rapid biochemical or immunological

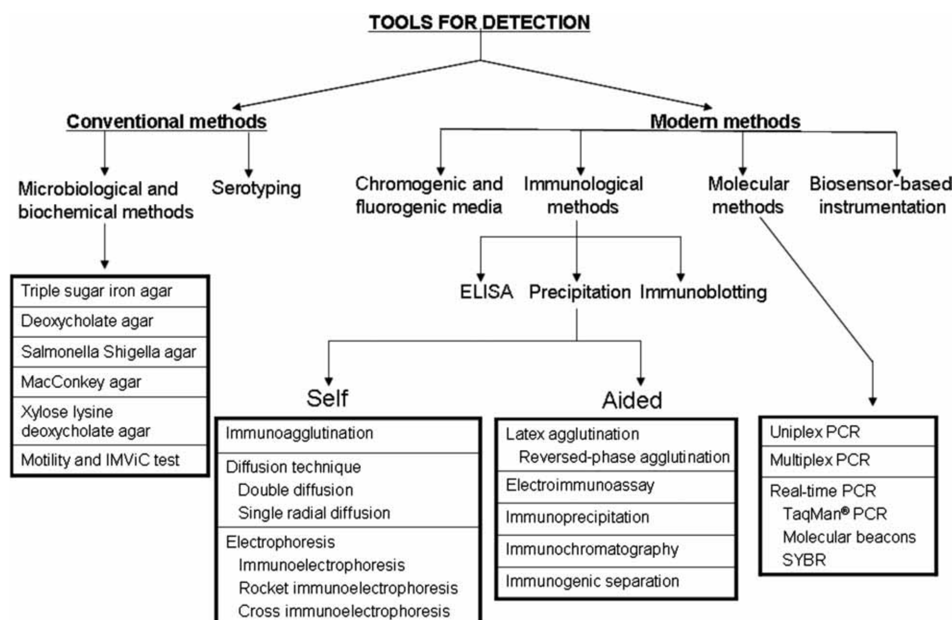

Fig. 6. Various types of screening and detection techniques of food pathogens

techniques based on functionally characterized and highly conserved virulence factors characteristic of a pathogenic *E. coli* (*96,97*). The following is an account of such a development that is currently taking place.

Conventional Methods

Microbiological and biochemical tests

E. coli pathogens from food samples can be easily recovered by growing in or on selective media, such as one that contains bile salts or other constituents that inhibit non-coliforms (Fig. 6, *98*). Though there are many enrichment media available, the International Commission on Microbiological Specifications for Foods (ICMSF) and International Standards Organization (ISO) have recommended the following method for the detection of *E. coli* in food products commonly used in food industries. The method involves two steps: bacterial growth in lauryl sulphate tryptose broth at 35 or 37 °C (mildly selective enrichment step), followed by growth in *E. coli* broth with 0.15 % bile salts at 45 °C (second selective step) (*99*).

Differential media allow *E. coli* to be identified amongst other bacteria by exploiting unique biochemical properties, such as the frequent production of a green metallic sheen on eosin methylene blue (EMB) agar, from lactose fermentation-based acid precipitation of dyes on the agar surface. Once *E. coli* is isolated, it can further be confirmed by a variety of biochemical tests, including the indole test for tryptophanase production, the methyl red and Voges-Proskauer (MR-VP) tests for mixed acid or 2,3-butanediol fermentation, respectively, citrate utilization, and motility (*100*). Distinguishing among pathotypes is problematic except for *E. coli* O157:H7, which is one of the strains negative for sorbitol fermentation, as shown on sorbitol MacConkey agar (SMAC) (*101*). Such methods are quite useful, but require adequate knowledge of differential biochemistry among the pathogenic types (*100*). Presence of unique and novel virulence-associated enzymes, transporters or binding proteins on the surface might also be utilized for such an approach. With increasing interest in proteomics, such surface-associated biomarkers can be identified in the research laboratories and exploited to develop simpler diagnostic tests. Recently, our group has developed a colorimetric procedure based on a virulence-associated periplasmic apyrase of EIEC to detect this pathogen and *Shigella* specifically from other *E. coli* (*102,103*).

Serotyping

Serospecific antisera hold the key to the success of this technique. Simple immunoagglutination on slides has actually helped to identify pathogens such as EPEC (*104*). Several variations of this principle exist, including the use of *Staphylococcus aureus* Cowan I cells (SAC) containing the abundant amount of protein A on the surface. Cowan I cells are grown in brain heart infusion (BHI), heat-killed, and suspended in 1 % PBS containing sodium azide. Cowan cells are absorbed with appropriate antisera for agglutinating the targeted bacteria. Such specific antibody-coated Cowan cells are useful agglutinating reagents to identify bacteria in clinical laboratories (*105,106*). Other immunological methods (see below) have been derived from this principle. The surface-specific antibodies help to resolve a given pathotype into an individual *E. coli* bacterium with a characteristic surface protein and polysaccharide profile (*104*). The most famous serotyping methodology for *E. coli* involves differentiating the organisms based on their O – somatic lipopolysaccharide (LPS) – associated carbohydrates, H – flagellar (protein) and K – capsular (carbohydrate) surface antigens. A specific combination of O and H antigens normally defines the 'serotype' of an isolate (*107*).

Serotyping is very useful in epidemiological surveys, but in cases where many serotypes cause a partic-

ular disease (as in the case of EPEC) this method becomes less reliable with one or a few antisera reagents and tedious if all the antisera have to be used. Serotyping is not economical and results are at times difficult to interpret and often require skilled personnel. Thus, this approach is not suitable for *E. coli* pathogen detection as part of many applications, such as food quality control. However, the same principle can be applied in a variety of immunological methods and formats described below.

Modern Methods

Growth in chromogenic and fluorogenic media

Use of enrichment media followed by growth in a medium containing a chromogenic or a fluorogenic substance that forms a coloured or fluorescent end product upon action by an *E. coli*-specific enzyme is becoming a preferred technique to identify common food pathogens. One example of such a method is the use of 4-methylumbelliferyl-β-D-glucuronide (MUG) incorporated into lauryl soy tryptose (LST) broth (*100*). The β-glucuronidase produced by *E. coli* cleaves the MUG substrate to yield a fluorescent end product. *E. coli*-negative samples can be identified by the lack of fluorescence in LST-MUG within 24 hours. This method has been successfully used for detecting EHEC, STEC and *Shigella* in many food products, which normally takes about 4–6 days to give very low false positives (*108*). Moberg (*109*) eliminated the enrichment step and used the MUG medium directly on 1400 food and dairy samples, while still achieving lower false positive rates (1.4 %) and saving precious time by reducing detection time to 2.5 days. The chromogenic tests are compatible with high-throughput screening and therefore have a good future. However, such contemporary methods are useful to detect pathogens only if they are linked to particular virulence determinants, otherwise commensal and nonpathogenic *E. coli* will also be detected.

The drawback of classical microbiological and biochemical methods as well as the advent of molecular biology has led to the development of many molecular techniques that could be used to detect *E. coli* in food samples and characterize pathotypes (*110*). These methods allow rapid and accurate detection of a variety of foodborne pathogens with outstanding sensitivity and specificity (*111*). An account of these is given below.

Immunological methods

Antigen-antibody reaction is the most common format for commercial kit-based detection of bacterial pathogens. Antibody-epitope specificity allows for development of strain-specific diagnostic assays. One of the earliest and simplest of the immunodetections of pathogens is slide agglutination test, successfully employed to decrease incidence of EPEC outbreaks in developed countries between 1950 and 1980. Even today it is popular in microbiology labs of developing countries. However, it suffers from poor sensitivity requiring dense bacterial cultures, which also interferes with interpretation of clumping.

Agglutination methods

Latex agglutination and Cowen I agglutination methods described above are variations of this simple and highly affordable technique with each sample costing $1-2. The simplest of the commercial immunoassays is latex agglutination (LA), using antibody-coated coloured latex beads or colloidal gold particles (*112*). This method is routinely used for rapid serological identification or typing of bacteria for most of the food pathogens, such as *E. coli* O157:H7, STEC, *Salmonella*, *Listeria* and *Pseudomonas* spp. from food (*111,113*). Reverse passive latex agglutination (RPLA) is used in the identification of soluble antigens or the toxins produced by the bacteria (*114, 115*). LA has better sensitivity compared to simple agglutination, and is generally performed on a glass slide as microscopic agglutination tests (MAT). The contaminated food or infectious sample is mixed with antibody-coated latex beads to see clumps or agglutinations within minutes (*116*). Though normally performed as one or a few samples at a time, Gerber *et al.* (*117*) proposed a microtitre plate LA for histoplasmosis as a method to detect several samples at a time.

Reverse passive latex agglutination (RPLA) for toxin or soluble antigen detection is more rapid and less ambiguous as soluble preparations are used and the clumping is easier to recognize. This method is routinely used in diagnostic laboratories and food industries for quality control purposes. A number of companies like Denka Seiken Co. Ltd., Japan, and Applied Biosciences International, USA, are providing ready commercial RPLA kits for a wide variety of toxins including Shiga toxin, verotoxin, cholera enterotoxin, *etc.* for mass screening. When compared to ELISA (see below), this is much more cost effective and time saving and hence preferred. In fact, due to weak cross-reactions because of food contaminants, ELISA results should be confirmed by the MAT results (*118–121*).

Though simple and less expensive, immunoagglutination may not be preferred where the antigenic variation is high and necessitates a diverse selection of antisera as in the case of serotyping based on O-antigens. Highly conserved and abundant surface-associated virulence proteins as diagnostic targets are superior in this regard. This approach is now becoming popular with the advent of molecular pathogenesis and large scale genomic studies. In a joint European Commission project to identify EPEC in stool samples and in food items, we are developing a simple immunoagglutination assay using antibodies for cell-surface virulence proteins, which are better conserved when compared to surface carbohydrates.

Enzyme-linked immunosorbent assay (ELISA)

It is the most widely used antigen-antibody assay in clinical diagnostics (Fig. 7). ELISA is normally performed in microtitre plates, although specialized variations exist, such as immobilizing the antigen/antibody complex to a solid support (nitrocellulose/nylon membranes) in order to increase sensitivity. In the antigen or pathogen detection mode, the antibodies specific to an antigen present on the organism to be detected are applied to the microtitre wells. The antigens from the food sample or enrichment culture are captured when they

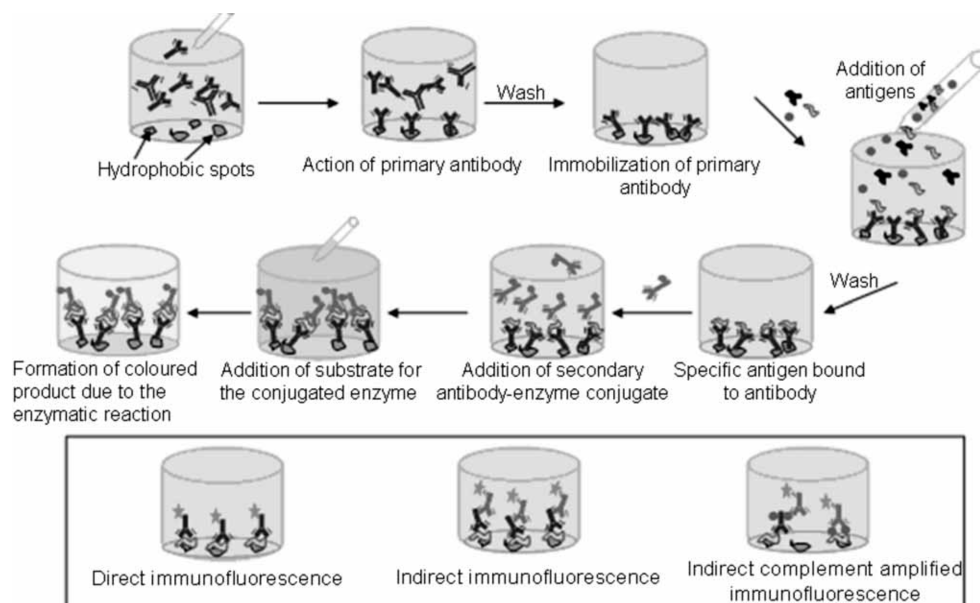

Fig. 7. ELISA and its variants

react with these immobilized antibodies. Either a primary antibody conjugated with enzymatic or fluorescent tag or, for better amplification of the signal, a secondary antibody to the primary conjugated with an enzymatic label (usually, alkaline phosphatase or horseradish peroxidase) or a fluorescent tag (*i.e.* fluorescein isothiocyanate) is used to obtain the signal (*122*,*123*). In another ELISA variation, conjugated protein A fluorescent dyes or colloidal silver/gold particles are used in place of secondary antibody. Conjugated protein A binds to the Fc portion of the IgG molecule and appears coloured or fluorescent (*124*). An ELISA approach has been utilized in our laboratory for detection of *Shigella* and EIEC exploiting the virulence-associated phenomenon of Congo red-induced secretion of invasion proteins by these pathogens (*125*,*126*). This requires growth of the organisms from stool samples to late log phase, facilitating detection even when cells are present in small numbers. Although ELISA is conceptually simple and automatable, sensitivity is several orders of magnitude lower than nucleic-acid-based approaches. Most of the existing ELISA-based methods can detect 1–10 μg/mL of antigen. Generally, microtitre plate made of polyvinylchloride (PVC) is used as it binds 1 μg/well (300 ng/cm^2) of antigenic proteins/antibody, which is several times higher than the sensitivity of the method. Normally, 50–100 μL of antigen/antibody solution (20 μg/mL in PBS) are employed for each well for binding (*127*).

Bohaychuk *et al.* (*128*) compared the efficiencies of ELISA, PCR and lateral flow immunoprecipitation with conventional culture methods to detect food pathogens like *Campylobacter*, *Listeria* and *Escherichia coli* O157:H7, and reported 80–100 % sensitivity with no statistically significant difference between these rapid methods and conventional culture methods in detecting *Escherichia coli* O157:H7 and *Salmonella*; PCR was found to be superior in detecting *Campylobacter* and *Listeria*.

Present methods for detection of food- and waterborne pathogens require a pre-enrichment step to selectively enrich the bacteria in order to bring them into detection limits. A flow-through immunofiltration method requiring no pre-enrichment has been developed to detect 100 cells/mL of *E. coli* in 30 min. However, clogging is a major problem while working with food samples (*129*). By concentrating bacteria or proteins during the flow of the sample through antibody-immobilized large (3 mm diameter) glass beads (to prevent clogging) and then performing ELISA test, Weimer *et al.* (*130*) eliminated the enrichment step and developed a method to identify bacteria (*E. coli* O157:H7), spores (*Bacillus globigii*) and small proteins in 30 min. This method claims detection of <10 bacterial cells/sample and 1 spore per cell.

A variety of solid matrices like polystyrene, polypropylene and polyvinylchloride are commonly in use and these are supplied by global manufactures and suppliers like Sigma-Aldrich Chemicals Pvt. Ltd. (St. Louis, MO, USA), Pierce Biotechnologies Inc. (Rockford, USA), and GE Healthcare (Buckinghamshire, UK). Fully automated rapid and mass screening assay kits for food pathogens are expensive but available on the market, *e.g.* from Molecular Circuitry Inc. (King of Prussia, PA, USA), Organon Teknika B.V. (Boxtel, The Netherlands) and Tecra International, Sydney, Australia (*130*,*131*).

Immunoprecipitation

Immunoprecipitation is a useful property of antibodies because it forms large complexes with multivalent antigens, which then precipitate from the solution. When antigen and antibody solutions are overlaid or their zones are made to merge, as in a solid-phase diffusion setup, precipitin discs and lines become visible. The precipitation techniques are generally performed using 2–3 mm thick agar gels cast on microscope glass slides with separate wells cut out for antigens and antibodies (*131–134*). Where possible, immunoprecipitation can reduce the time and labour in screening and detection of pathogens (*128*).

Double diffusion method

There are interesting and useful variations to classical immunoprecipitation techniques combining diffusion and electrophoresis. In simple diffusion techniques, such as the popular Ouchterlony double diffusion, wells for antigens are punched around a central well containing antibody. As the antigens and antibody diffuse radially from the respective wells, their zones merge and at a particular point precipitation occurs, which appears as opaque line or arc. These precipitin lines can be stained with Coomassie Blue or Amido Black after washing away soluble complexes and unreacted antigens and antibody (Fig. 8). The antigenic differences can be deduced by the non-overlapping nature of the lines; smooth merger of the precipitin lines at the ends indicates the same antigens. This simple method works well in the range from 20 μg/mL to 2 mg/mL of antigen and antibody (*132,133,135*).

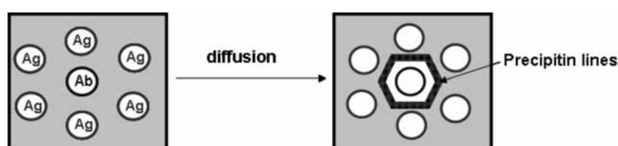

Fig. 8. Double disk diffusion

Radial diffusion method

In the radial diffusion technique, the gel matrix is cast with antibody in it and the antigens are placed in wells and allowed to diffuse. The precipitin zone forms a circle around the well and its annular width indicates similarities between various antigens (Fig. 9). This technique is useful for determining concentrations of antigens such as serum proteins. For example, lactoferrin concentrations in the cow's milk were analyzed by Hagiwara *et al.* (*136*). They reported that 200 μg/mL of lactoferrin are required to resist the growth of *E. coli* pathogens. The agar gel precipitin (AGP) test, based on an immunoprecipitation principle, was used to detect *S. enteritidis* antibodies present in egg yolks of infected hens. The experiment was performed by loading infected yolk and its antibody in the agar gel slab to precipitate. This is generally used to study multiple avian pathogens, but requires large amounts of antigen and antibodies as they are examined on agar bases (*132,133,137*).

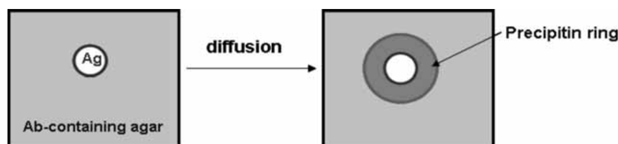

Fig. 9. Single radial diffusion

Immunoelectrophoresis

Diffusion is a slow process and therefore to aid quicker formation of the zones, electrophoresis is used. The ability of electrophoresis to separate complex antigenic mixtures is also exploited. In simple immunoelectrophoresis, the antigenic mixture is electrophoretically separated in one dimension, as shown in Fig. 10. A well for the antibody is cut parallel to separate antigens and both are allowed to diffuse. Precipitin lines are formed as in the case of diffusion methods. The advantage of this method is simultaneous analysis of multiple antigens in a mixture like patient's serum, concentrated urine or spinal fluid.

The principle of rocket immunoelectrophoresis (RIE) involves electrophoretic migration of antigen from wells cut in an antibody-containing gel (Fig. 11). This saves the time of diffusion as the antibody is already present in the electrophoretic medium. However, since directional movement of antigen is involved, the appearance of precipitin line would resemble the outline of a rocket, hence the name. The pH of the gel is chosen to prevent antibody mobility. As the height of the rocket is proportional to antigen concentration, it is useful to find out and compare antigen concentrations. The sensitivity of this method is 10- to 20-fold higher than that of simple diffusion techniques.

Crossed immunoelectrophoresis combines the benefit of electrophoretic separation of complex mixtures of antigens in the first dimension and that of rocket electrophoresis in the second dimension to obtain characteristic immunoprecipitation lines (Fig. 12). An obvious

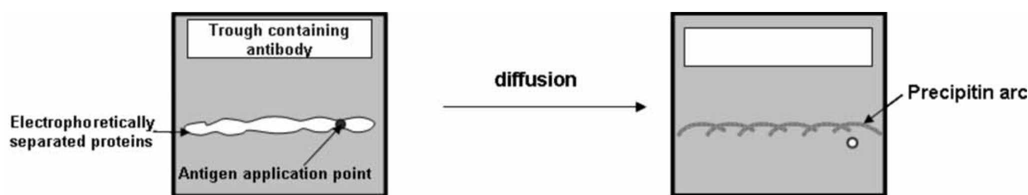

Fig. 10. Immunoelectrophoresis

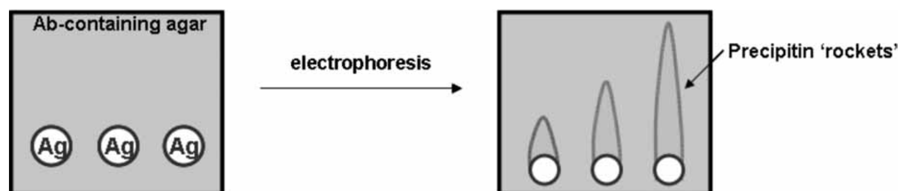

Fig. 11. Rocket immunoelectrophoresis

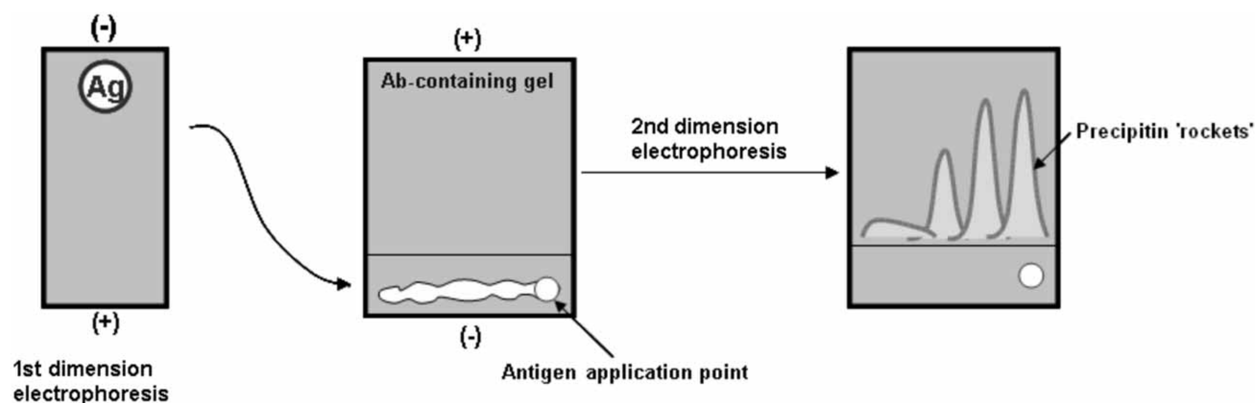

Fig. 12. Crossed immunoelectrophoresis

disadvantage of such electrophoretic techniques is the relatively poor resolution of antigen mixtures using agarose gel electrophoresis (*132,133*). The study of a foliar fungal pathogen in tea by Dasgupta *et al.* (*138*) provides a good example of the use of immunodiffusion, immunoelectrophoresis and ELISA to screen antigens. Holzhauser *et al.* (*139*) reported detection of 20 ng/mL of peanut protein when analyzed by RIE for food allergens.

Immunoprecipitation of soluble Ag-Ab complexes

When the immunocomplexes are soluble, they can still be precipitated with the aid of immobilized secondary antibodies or protein A attached to insoluble particles. Such precipitants can be heat-killed *Staphylococcus* cells (Cowan cells) with protein A on the surface, or protein A/secondary antibodies immobilized to beads made of agarose, latex, polystyrene, or the like. Unlike self-precipitation of Ag-Ab complexes, this indirect precipitation method offers higher sensitivity and the advantage of a simpler approach. Radioimmunoassays (RIA) and radio-immunoprecipitation assays using radioactive antigens is a popular laboratory technique for quantifying minute quantities of biologicals such as hormones.

In the simplest form of immunoprecipitation of soluble antigens, latex agglutination or colloidal gold agglutination are popular techniques. Food samples containing antigen are applied to antibody-coupled coloured latex beads or colloidal gold. Visible clumping of the coloured beads or particles indicates the presence of a target antigen, such as a toxin. Such tests are popular because they are completed within minutes, but require enrichment of antigens (*140,141*). In our Centre, a rapid (3 min) screening kit for early detection of filariasis using a pathogen-specific protein, WbSXP-1, has been developed. When the test serum is passed through a horizontal flow-through device, the antibodies in the sera bind to WbSXP-1 immobilized to a nitrocellulose disc. Colloidal gold protein A conjugate is then allowed to bind the antibodies showing a pink spot in the test area (*142*). Such methods can be easily adapted to detection of *E. coli* toxins or its secretory virulence factors, which have been identified recently.

Immunochromatography

This approach is based on reversible interaction between a covalently immobilized antigenic ligand and antibodies. Agarose, sepharose, polyacrylamide, polystyrene, and cellulose are common matrices for the attachment of antigenic ligands, which are coupled using their primary amines, carboxyl, hydroxyl or thiol groups. When the antibody-containing solution is passed through the affinity matrix, the antibody binds the ligand. The antigen-antibody complex is broken by acidic buffers such as glycine-HCl (pH=2.2) and the antibody is eluted. This type of immunoaffinity chromatography is popular to purify specific antibodies to a ligand or even IgG monoclonal antibodies using immobilized proteins A, G and L. With immobilized antibodies, immunoaffinity chromatography is also popular in single-step purification of antigens. A recent report documents purified legumin from *Pisum* (pea) types (*143*). In diagnostic use, the same principle may be applied but restricting the immobilization of antigen/antibody to a dot, line or a zone on a strip or disc made of nylon, nitrocellulose, cellulose *etc.* is used to detect specific antibody or antigen respectively. Visualization is done using reporter-tagged secondary antibody.

Electroimmunoassay

Electroimmunoassay (EIA) is based on deriving an electric signal directly from a specific antibody-antigen reaction. Antibodies are attached to the solid surface of one of the electrodes and when the sample is applied, the target antigen binds to the antibody. Subsequently, colloidal gold–labelled secondary antibody binds and the complex helps to bridge the electrodes, especially when silver ions are deposited onto the colloidal gold. The conductive silver bridge closes the circuit and causes a drop in resistance that is measured. Detex MC-18 system developed to detect *E. coli* O157:H7 is based on this principle (*144,145*). This expensive device has been used for rapid screening of C-reactive protein (CRP), a marker for inflammation that plays a major role in neonatal septicemia, meningitis, otitis and cancer. In urinary tract infection (UTI), EIA has been used along with semi-quantitative latex-agglutination (*146,147*). Zhou and Chang (*148*) incorporated carbon nanotubes and gold electrodes and demonstrated the screening of <10 000 cells/mL of bacteria.

Nucleic acid methods

Polymerase chain reaction (PCR)

Polymerase chain reaction, popularly referred to as PCR, has become a hugely popular and essential technique in research laboratories (Fig. 13, *149*). The ability of PCR to amplify billions of copies of a given DNA sequence from a heterogenous starting sample has made it a popular tool for screening and characterization of pathogens. The major drawback of PCR as a clinical diagnostic tool, however, is the requirement for skilled personnel and well standardized and tested primers and protocols. Media components like calcium ions, casein hydrolysate, along with a myriad of other antagonists, inhibit assay sensitivity. Template quality is a critical factor, which varies with samples and requires skill and precision. Notwithstanding these practical limitations, PCR-based detection is a successful and widely used diagnostic tool in clinical laboratories. Many primers are now available for accurate and sensitive detection of various pathotypes of diarrhoeagenic *E. coli* (*150*). Sensitivity of PCR is the main factor that makes it desirable as a detection tool in food industry. Though setting up a PCR facility might be costly in the beginning, once established, the test cost could come down to moderate levels. Methods to detect more than one probable pathogen as in the case of multiplex PCR (see below) can cut down this cost further. Hence PCR-based methods are being developed for detection of pathogens in food samples.

Multiplex PCR

In this method, pairs of primers specific for genes of different pathotypes are used in a single PCR reaction for detecting one or more of these targets simultaneously (*151*,*152*). Multiplex PCR is conceptually an efficient method as it allows both detection and characterization of a few to many pathogens in a single step reaction. Typically, detection of no more than four target genes or sequences is readily possible. Unlike uniplex PCR setting, a multiplex reaction can be difficult because of increased complexity with respect to optimized primer combinations and reaction conditions. Sample mixtures are normally amplified in 20- to 50-μL reaction mixture containing 10–100 ng of DNA. Multiplex PCR is likely to be less sensitive than uniplex PCR, owing to the complex reagent dynamics with multiple primer pairs in the same reaction tube. A number of recent reports demonstrate results with minimal false negatives (*153–155*). In our lab, we have been using successfully multiplex PCR primers and conditions reported by Kong *et al.* (*152*), and Aranda *et al*. (*156*) for characterizing clinical *E. coli* strains isolated from diarrhoeal samples. Watterworth *et al.* (*157*), Vidal *et al.* (*158*) and Kimata *et al.* (*151*) reported similar use of multiplex PCR to screen and categorize *E. coli* pathotypes.

Real-time PCR

Real-time PCR (RT-PCR) is another popular method for detection of bacterial pathogens in food. Real-time PCR enables one to monitor the progress of PCR as it

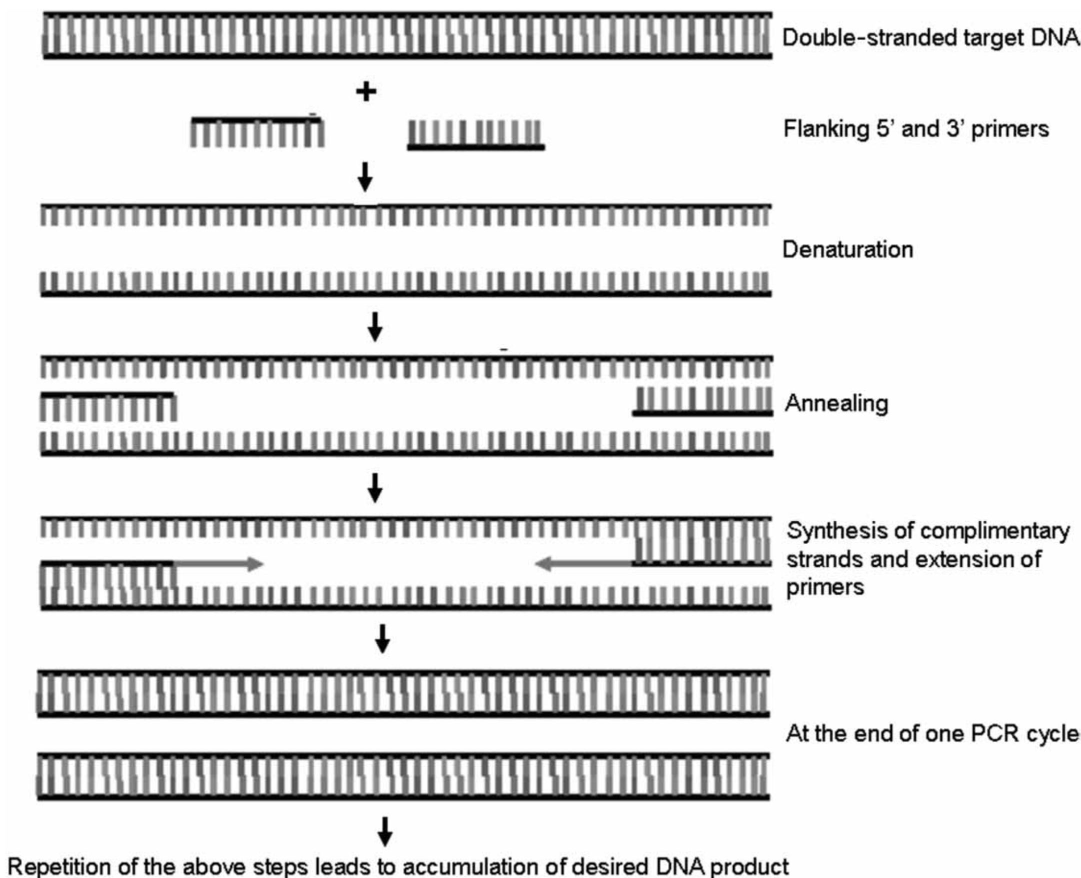

Fig. 13. Polymerase chain reaction (PCR)

occurs by measuring fluorescence associated with the product (*159*). In traditional PCR, the amplified product is detected after cycling using agarose gel electrophoresis and ethidium bromide staining. Since endpoint detection does not accurately assess relative DNA (starting) amounts, such an approach is limited for qualitative studies and is obsolescent. Real-time PCR allows such quantification by correlating fluorescence during progress of the reaction to the amount of DNA template; with higher copy number templates, fluorescence can be detected sooner. This *in situ* quantification ability has allowed automation in high-throughput formats. A variety of real-time detection chemistries are available that differ in cost, sensitivity, and ease of use. The most useful among them are SYBR Green probes, TaqMan probes and molecular beacons (FRET based methods). Tian and Mandrell (*160*) developed a sensitive real-time immuno-PCR (rtI-PCR) method which is more than 1000-fold more accurate than the standard ELISA for the detection of Norwalk-like viruses (NLV) in food samples using biotin conjugated DNA reporters and incorporating multiple washing steps to eliminate the PCR inhibitors.

SYBR Green-I (SGI) dye. This compound is a dye that fluoresces upon binding to the minor groove of double-stranded DNA. Therefore, when it is present in a PCR reaction, its fluorescence intensity varies as a function of amplification (Fig. 14). Owing to this property, SGI has been used in quantitative PCR (qPCR), developed by Higuchi *et al.* (*161*), as well as in quantifying RNA copies in a cDNA reaction. Giglio *et al.* (*162*) performed a real-time multiplex screening of *Vibrio cholerae* and *Legionella pneumophila*. As it is relatively inexpensive, SGI is preferred in diagnostic real-time PCR when compared to other expensive fluorescent probes. It is also compatible with agarose gel electrophoretic analysis (*161*,*162*).

TaqMan® PCR. TaqMan® is a sequence-specific probe of 20–30 bp labelled with a fluorescent reporter dye on one end and a fluorescent quencher on the other (Fig. 15). During PCR, this probe, flanked by the sequence-specific primers, binds to the target region. When polymerization occurs, the probe is degraded by the 5'→3' exonuclease activity of *Taq* DNA polymerase. This causes separation of the reporter dye from the quencher dye, eliciting a detectable fluorescent signal. Fluorescence increases exponentially as cycling progresses with fluorescence data acquisition occurring in real time at each extension cycle. Applied Biosystems (Foster City, CA, USA) has developed a TaqMan® PCR method for detection of *E. coli* pathogens. This method has also been successfully used for characterization of 54 foodborne *E. coli* strains by Davis *et al.* (*163*).

Molecular beacons. Molecular beacons are short sequences with a stem-loop secondary structure containing a fluorescent reporter conjugate on one end and a quencher moiety at the other end (Fig. 16). When not hybridized to the target sequence, the quencher is close in proximity to the reporter and no signal is observed because the resonance energy transfer occurring between the conjugates is released as heat rather than light energy. When the molecular beacon binds to the target sequence, the fluorophore and quencher are separated as the molecule opens, and fluorescence is observed. In a study by McKillip and Drake (*164*) this method was used to detect *E. coli* O157:H7 in milk samples. Using an appropriate probe to the *stx2* gene coding for the A subunit of the cytotoxin, the degree of fluorescence in PCR reactions was correlated with bacterial load of 10^3, 10^5 and 10^7 CFU of *E. coli* O157:H7 per mL.

It should be cautioned that though these real-time PCR techniques are superior to traditional and quantitative PCR, the initial investments in instrumentation and infrastructure could be as high as $100 000 and the running costs could be $20-50 per sample depending on the chemistry. Currently, these are critical factors weighing heavily against such sophisticated techniques while considering practical applications of routine diagnosis at an affordable cost. The power of real-time PCR techniques

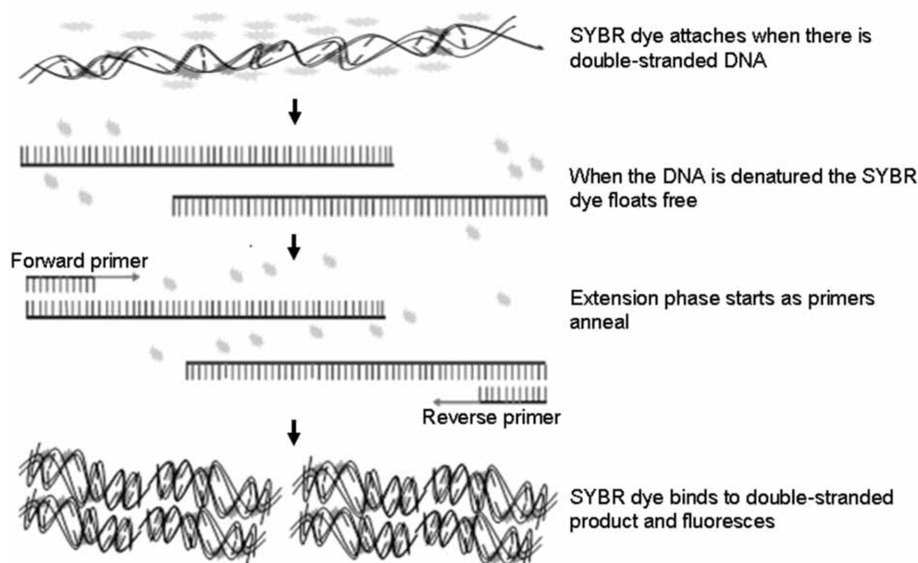

Fig. 14. SYBR amplification

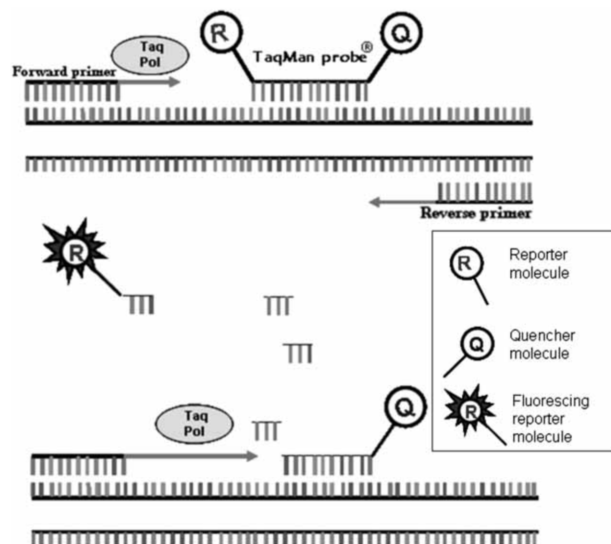

Fig. 15. TaqMan® probe

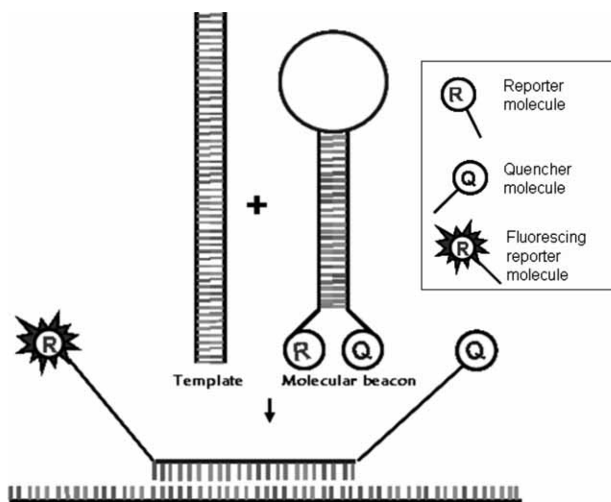

Fig. 16. Molecular beacon

has been demonstrated in applications of viral quantitation, quantitation of gene expression, array verification, drug therapy and its efficacy, DNA damage measurement high throughput pathogen detection, and genotyping. There are many ongoing attempts to develop application-specific low cost instrumentation and reagents.

Cell concentration methods to aid sensitive detection

All available analytical methods, except for microscopy, require minimum number of bacteria, normally in the range of 10^4-10^8, which means they have to be enriched if present in lesser numbers in the sample. This requires either the concentration or culturing of bacteria for a few to several hours, especially for early and sensitive detection. One of the simple and popular methods is the filtration of sample through bacterial filters to capture bacterial pathogens. This is normally used in identifying coliforms including *E. coli* in water sources. The filtration method is not suitable for particulate samples like food materials, as fouling of pores in the filter is a major problem.

Immunomagnetic separation

Immunomagnetic separation (IMS) is a recently developed method that has been adapted for detecting food pathogens (*165–167*). This method uses magnetic beads coupled to antibodies directed toward antigens on bacteria. Solid-phase laser cytometry is one means of subsequently assessing and enumerating viable bacterial populations in water and food. Pyle *et al.* (*165*) used IMS for food pathogen detection. Enumeration by laser scanning permitted detection of 10 CFU/g of ground beef or <10 CFU/mL of liquid sample. They used IMS and flow cytometry on solid phase to detect *E. coli* O157:H7 in water and food samples after incubating bacteria with cyanoditolyl tetrazolium chloride (CTC) to detect the respiratory activity of the bacteria. This method requires enrichment/incubation with super-paramagnetic beads coated with anti-O157 rabbit serum with a sensitivity of >10^4 cells/g. About 90 % specificity was reported when they compared the sensitivities of IMS and PCR. IMS can be applied in the screening of widespread detections of O157:H7 in clinical and food microbiology laboratories as it is less labour-intensive, less time-consuming and 90 % sensitive compared to the molecular methods like PCR (*166*).

As an alternative to IMS, metal hydroxides have been helpful in immobilizing and concentrating bacteria, thus aiding their detection (*168*). Zirconium hydroxide and hafnium hydroxide have been used to detect *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* in reconstituted non-fat dry milk. At pH=7.2, zirconium chloride in water forms multiple tetrameric complexes in which zirconium ions are connected by hydroxide bridges. The gelatinous precipitate (zirconium hydroxide) forms effective covalent bonds between the hydroxyl groups of the metal hydroxide and the amino acid side chains that are abundant on the bacterial cell surface and non-specifically immobilize and increase the bacterial concentration up to 50 fold (*169*). The immobilized bacteria can then be analyzed by any one of the techniques, from conventional microscopic methods to RT-PCR (*170,171*).

Biosensor-Based Instrumentation

The need for surveillance of *E. coli* pathogens cannot be underscored enough (*172*). For effective surveillance, the methods should be rapid, sensitive, specific, and more importantly, capable of high throughput. In food samples, confirmation of cell viability and detection of virulence determinants is crucial (*173*). Furthermore, some of the food constituents or carryover components from processing steps are known to inhibit enzymatic reagents and antibody reactions. These have to be effectively removed with minimal manipulations. Growth of bacteria from food samples (*i.e.* enrichment steps) prior to detection is convenient but causes delay (hours to days). It is a trade-off for sensitivity, specificity and cost. Enrichment steps may also help to enhance the expression of virulence factors; for example, growth of EIEC and *Shigella* in presence of Congo Red or presence

and absence of Fe and Ca in the expression of some of the toxins of *E. coli* (*125,174*). Growth step also helps dormant life forms like spores to germinate and provide vegetative forms for detection.

Among several methods available for detection of pathogens, optical signals, either as colour or fluorescence, from biospecific antigen-antibody reaction converted to electrical output using devices like sensitive photodiodes (optical biosensor devices) offers the best design for instrumentation that is affordable to peripheral labs (*174*). There are other expensive approaches using plasmon resonance and electrochemical measurements after antigen-antibody reaction or DNA amplification. Such instruments already on the market are not used for *E. coli* detection and need economical adaptation.

Generally, plasmon resonance biosensors (PRB) (*175*) are used to detect food pathogens like *E. coli*, *Salmonella*, *etc.*, using immobilized protein A, protein G and further reactions with specific antibody and have the potential to detect up 10^7 CFU/mL of cells (*176,177*). Researchers at Cornell University, USA have developed simple and efficient biosensors for *E. coli*, dengue virus and *Bacillus anthracis.*

Realizing that instrumentation for infectious diseases is neglected in countries like India, our group is currently developing an immunofluorescence-based detection device that can overcome routine conventional procedure in the detection of infectious pathogens. The immunospecific binding of pathogens is detected using fluorescent probe (with Ex/Em in visible range) as a voltage output. Biosensor-based instrumentation development is an emerging field that may provide a means of surveillance of dangerous pathogens like *E. coli.*

Concluding Remarks

Recent discoveries on the pathogenic potential of *E. coli* are turning our attention to the need for heightened surveillance of various pathotypes. The ability to acquire new virulence determinants including multi-drug resistance by horizontal gene transfer coupled with the abundance of pathogenic strains complicates surveillance. Recent advancements in molecular pathogenesis of a variety of *E. coli* serotypes is helping to devise appropriate methodologies and instruments to directly address early detection and surveillance. Food is a major vehicle for spreading *E. coli*-mediated diseases, and thus it is imperative that pathogenic *E. coli* detection should become an essential feature in food processing.

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