

## Molecular and Microbiological Studies of Food Products to Detect Microbial Contamination

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**Abstract:** Foodborne diseases are a major risk for human health. Millions of people get sick as a result of eating contaminated food. Food poisoning occur due to consumption of water and food which is contaminated with microorganisms that cause diseases. Food poisoning is suspected when an acute illness with the adverse effects on the digestive system or the nervous system, affects two or more persons, who shared the meal during the past 72 hours. In microbial food poisoning, microbes multiply in the food before consumption, while in the food-borne infection, food is just a carrier for the microbes as they do not grow on the substrate. Early detection of food-borne pathogens with high specificity and improved sensitivity is very crucial, such as in case of an outbreak of foodborne illness. The traditional detection methods are less sensitive, laborious and time-consuming. Molecular detection methods based on latest genomic technologies are more sensitive, such as next-generation sequencing, Real-Time PCR, microarray and others molecular biology techniques are able to provide faster results with more accuracy. DNA based testing is now feasible on a single molecule and high-throughput screening methods facilitate analysis of various parameters simultaneously, thus allowing various characteristics to be determined quickly and with more sensitivity. The purpose of the present review is to elucidate the application of modern DNA technology for detecting food contaminations by analyzing the genetic material in the sample, data analysis and highlights various testing procedures with high specificity and improved sensitivity for monitoring pathogens in food items.

**Key words:** Food products • Foodborne diseases • DNA technology • Diagnosis • Infection

### INTRODUCTION

Whole genome sequencing to compare the DNA sequences of bacterial chromosomes would be ideal to determine food contamination, but it would be very expensive and time consuming process. There are several other molecular biology techniques such as DNA hybridization, sequencing of the most common gene encoding for 16S rRNA and polymerase chain reaction (PCR) which can be exploited to identify microorganisms in food [1, 2]. The main advantage of PCR technology as compared

to other methods, is in saving time, as starting from sample collection to data analysis is completed in a very short span of time.

The major food born disease causing microorganisms are bacteria such as *Salmonella*, *Shigella*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus* and *Clostridium botulinum*. Bacteria present in food items cause disease in humans either by infecting directly or due to the actions of the toxins that were released by pathogens before or after the food intake [2-4]. Viruses generally cause disease by infecting directly. The most dangerous viruses detected in contaminated food

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items are hepatitis virus (HAV) and Norwalk -type virus. The microbial contamination can occur in the food prepared or delivered by infected workers.

Cocolin *et al.* [5] developed a multiplex PCR based method to detect enteropathogenic and enterohemorrhagic *E.coli* in contaminated food. Lindstrom *et al.* [6] also used multiplex PCR to detect *Clostridium botulinum* type A, B,E and F in contaminated food as well as fecal matter. This method was very sensitive and specific in screening various serotypes of *Clostridium botulinum* and provided a significant improvement in the diagnosis of *C. botulinum* in contaminated food. Besides bacteria, many fungal species also grow on food products and spoil them particularly in case of fruits and vegetables. However, some fungal species have beneficial affects too [7, 8,9]. the tracking of *E. coli* O157: H7 in food items, clinical samples and environmental samples which is required for the diagnosis of infection. Revealed that *Salmonella enteric* (Satan) gene is highly conserved and the method developed in this study can be used as a quick and reliable procedure for monitoring *Salmonella* in water, milk and blood samples. Identified various molecular markers for identification of pathogenic *E. coli* strains belonging to serogroups O138 and O139 and these were found to be very accurate and extremely sensitive and fast. This method has been proposed as a new diagnostic tool to investigate *E.coli* infection and the spread of these pathogenic strains in animals and humans [10].

**Microbial Studies:** Foodborne and waterborne infection due to various bacterial pathogens are a major cause of mortality and morbidity in developing countries. Many developing and poor nations lack sufficient resources to provide hygienic facilities and safe water for consumption. The major bacterial pathogens that are transmitted through contaminated food and water include strains of *Salmonellae*, *Shigella* (*S. dysnteriae*, *S. flexneri*, *S. sonnie*, *S. boydii*); *Escherichia coli*, *Vibrio* (e.g., *V. cholerae*, *V. parahemolyticus*, *V. vulnificus*); *Campylobacter*, *Yersinia* and *Staphylococcus*. The mechanisms of pathogenicity caused by these bacteria require synergistic actions of several virulence factors produced by the bacteria. In addition to the multiple virulence genes, many of these bacteria also possess the capability to adapt and survive in water for longer duration. Evaluating the epidemiology and pathogenesis of these bacterial pathogens would contribute significantly to prevent foodborne and waterborne infections [2].

**Microbiological Hazards:** Microbiological hazards are responsible for the most foodborne diseases globally. The three major hazards are bacteria, viruses and parasites which can cause one of the three types of illness i.e infection, intoxication, or toxin-mediated infection.

**Infection:** A foodborne infection occur when food containing harmful microorganisms is consumed. These microorganisms grow in the intestine and cause severe infection. Only some bacteria causes food borne illness through infection while most of the viruses and parasites trigger foodborne disease via infection only. Some of the bacterial strains that cause foodborne infection are *Salmonella* spp., *Vibrio parahaemolyticus*, *Campylobacter jejuni*, *Yersinia enterocolitica* and *Listeria monocytogenes*. The major viruses that cause foodborne infection are Rotavirus, Hepatitis A virus and Norovirus. The major parasites that cause foodborne infection include *Trichinella spiralis*, *Toxoplasma gondii*, *Anisakis simplex*, *Giardia duodenalis*, *Cyclospora cayetanensis* and *Cryptosporidium parvum*.

**Intoxication:** An intoxication occurs when food containing toxins is consumed resulting in sickness. Toxins can be products of pathogenic microorganisms, chemical contamination, or are components of a plant or seafood. Bacterial strains are generally responsible for intoxication in human while viruses and parasites are not known to cause foodborne intoxication in human. The bacterial strains that trigger foodborne intoxication are *Clostridium botulinum*, *C. perfringens*, *Bacillus cereus* and *Staphylococcus aureus*. Several chemicals such as pesticides, cleaning products, sanitizers and metals like lead, copper, cadmium can cause an intoxication in human. Seafood toxins such as ciguatera toxin, scromboid toxin, shellfish toxins and some varieties of mushrooms can also cause an intoxication in human.

**Toxin-mediated Infection:** A toxin-mediated infection can take place when food containing harmful bacteria is consumed. The bacteria produces toxins as soon as it enters the intestinal tract which cause sickness. Some bacteria are known to trigger toxin-mediated infection while viruses and parasites do not cause a toxin-mediated infection. The bacterial strains responsible for toxin-mediated infection include *Shigella* spp. and *Escherichia coli* strain which produces Shiga toxin [11].

**Isolation and Identification of Bacteria:** To isolate bacterial strains, samples of body fluids such as blood or urine are plated on culture dishes and incubated for

several days at optimum temperature. Single individual colony which consists of millions of cells are isolated and further characterized.

#### **Biochemical Confirmation and Serotyping of Salmonella:**

Biochemical tests are performed to confirm the *Salmonella* strain. The ISO-6579 standard (iso 6579,2002 (E)) recommends the use of the TSI agar, Urea agar (Christensen),  $\alpha$ -galactosidase (ONPG), Voges Proskauer test, L-lysine decarboxylase and Indole tests to identify *Salmonella* contaminations. It has been clearly shown that Triple Sugar Iron (TSI) agar, mannitol, urea, lysine decarboxylase and ornithine decarboxylase test would be ideal to confirm *Salmonella* contamination. After performing Gram staining, cells are observed under the microscope to verify the morphology of bacterial cells.

***Salmonella* as Example of Foodborne Illness:** *Salmonella* contamination is one of the major cause of food-borne diseases globally. The *Salmonella* family is comprised of over 2,300 serotypes, but two serotypes i.e. *Salmonella enteritidis* and *Salmonella typhimurium*, are responsible for causing more than half of all human infections. It is known that *Salmonella* can grow on any food items but most outbreaks of salmonella are due to contaminated dairy, poultry and meat products. chicken, eggs and several other products derived from them are particularly considered high risk. Microbial control in the food industry plays an important role in checking *Salmonella* infections. Various biochemical and microbiological tests and media used for identification and characterization of *Salmonella* take advantage of unique aspects of *Salmonella* physiology or biochemistry as compared to other genera within the Enterobacteriaceae family. *Salmonella* are mainly facultative anaerobes, gram negative rods, oxidase-negative, catalase-positive and most strains are motile and ferment glucose with production of acid and gas. Presently, the media used for the identification of *Salmonella* are based on the detection of carbohydrate fermentation, the detection of proteolytic activity, hydrogen sulphide production [12]. The major Pathogenic bacteria such as *Salmonella*, *Shigella*, *Staphylococcus aureus*, *E.coli*, *Bacillus cereus* and *Clostridium botulinum* cause sickness in humans by infection or due to the toxic activity of toxins produced before or after food intake [4, 12].

#### **Identification and Differentiation Media for Salmonella:**

The genus *Salmonella* is comprised of three species termed as *Salmonella enterica*, *Salmonella subterranean* and *Salmonella bongori*. The *Salmonella enterica* is

further classified into six different subspecies i.e enterica (subsp. I), arizonae (subsp. IIIa), diarizonae (subsp. IIIb), houtenae (subsp. IV), indica (subsp. VI) and salamae (subsp. II). There are more than 50 serogroups in *Salmonella*, based on the O antigen and there are 2500 serovars, each having a different composition of somatic O antigen, flagellar H1 antigen and H2 antigens. Many of these serovars approximately 1,531, belong to *Salmonella* subsp., *enterica* and are responsible to cause more than 99% of the infections in humans which also include gastroenteritis and typhoid fever. Whole genome sequencing and comparative analysis of 28 serovars of *Salmonella enterica* revealed extensive homology in core regions of the genomes and also detected recombination and rearrangement, pseudogenes, genomic degradation and clonal diversity among the serovars. Comparative analysis of genomes of host-restricted *Salmonella* strains such as *S. Typhi*, *S. Paratyphi* and host-adapted *Salmonella* strains such as *S. Typhimurium*, *S. Enteritidis*, suggested that genomic degradation is an important evolutionary mechanism for survival and adaptation inside the host and also assist in increased pathogenicity of *Salmonella*. Hotspots for development of drug resistance in *Salmonella* are found to be localized in *Salmonella* genomic island 1 which harbor several antibiotic resistance gene clusters and conjugative R plasmids which are known to confer resistance to several common antibiotics including broad-spectrum cephalosporins. It has been suggested that genetic rearrangements in *Salmonella* leads to increased virulence and the development of resistance to multiple drugs [13].

Conventional culture methods for identification of pathogenic bacteria such as *Salmonella* in food samples require inoculation of the food sample into a nutrient medium in which the microorganisms can grow and multiply. These traditional methods are very simple, easily adaptable and very cost-effective. However, these methods depend on the growth of the microorganisms in different culture media, which may require several days before results are known. Hence these conventional methods cannot be used for food products that are minimally processed as they have a very short shelf life. In the last decade, extensive research has been done to reduce the detection time by developing novel and alternative methods for identifying foodborne microorganisms and also reduce the manual labor by developing high-throughput automated methods [14-17].

**Bacterial Contaminations in Powdered Infant Formula Products:** Powdered infant formula products are generally not sterile and thus may be contaminated with

microorganisms [18]. During the last two decades, there have been several reports of bacterial contamination of infant formula which caused severe infection in infants who have been fed with these products. The occurrence of gastrointestinal symptoms and bacteremia has been observed due to consumption of contaminated nutritional formula [19]. There are now sufficient evidences that revealed phenotypic and genotypic similarities between bacterial isolates obtained from infected human and those recovered from nutritional formula given to patients [20]. *Salmonella* species and various other members of the family *Enterobacteriaceae* have been detected in most of these cases of infections [21].

Two FAO/WHO meetings of experts held on the microbiological safety of powdered infant formula evaluated various cases of sickness in infants caused by formula consumption [22]. They identified three different categories of microorganisms based on the evidence of an association between their presence in prepared formula and infection caused by these bacteria in infants: one category includes bacteria such as *Salmonella enteric* and *Enterobacter sakazaki*, which exhibit clear evidence of causality. Another category include bacteria for which the causality is possible but not verified clearly yet. Although these pathogens are known to cause infection in infants and also have been detected in prepared formula, but contaminated formula has not been convincingly shown to be the main source of infection. *Salmonella* serotype Tennessee belongs to this category as it caused illness in infants in the United States and Canada and was supposed to be due to the intake of contaminated powdered milk product, which was later on recalled from the market after several cases were detected [23]. In another incidence which occurred in Spain in 1994, around 48 cases of *Salmonella* infection among infants were linked to intake of powdered infant formula which was found to be contaminated with *Salmonella Virchow*. Bornemann *et al.*, [24] reported an outbreak of *Salmonella* Serotype Saintpaul in a Children's Hospital after consumption of infected infant formula. Detection of *E. sakazakii* in dried milk products has been shown to be associated with development of disease among infants. These microorganisms generally cause septicemia, meningitis and urinary tract infection in patients [25]. It has been reported that contamination of infant formula with *E. sakazakii* caused necrotizing enterocolitis among infants in a neonatal ICU in Belgium [26]. It has been suggested that increased resistance to heat in *E. sakazakii* may play a role in its ability to colonize dried milk. Marino and colleagues conducted a study to evaluate the prevalence of bacterial contamination in

prepared infant formula given to infants at Red Cross War Memorial Children's Hospital, Cape Town. Their study revealed that even when milk is prepared in a controlled aseptic environment, there is still significant bacterial population in prepared infant formula post- production [27]. Their study indicated that 30% of prepared infant formula analyzed, including freshly prepared products, were contaminated with bacteria. It has been suggested that bacterial contamination in infant formula may occur during mixing and addition of additives and also during the initial storage.

#### **Outbreaks of Food-borne Infections in Hospitals:**

Contaminated milk has been implicated in food-related outbreaks of gastroenteritis in hospitals. This is one of the major concern as there is large scale consumption of milk in the hospitals (American Hospital Association, 1983). Most hospital associated food- borne infections are found to be caused by food items prepared by the health workers [28]. Nosocomial outbreaks due to contaminated infant formula have been reported globally with various pathogens such as *Enterobacter* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp., *Escherichia coli* and *Salmonella* spp. as the main causative agents [19]. Besides, *Enterobacter sakazakii* has also been identified as a pathogenic contaminant of milk products It has been speculated that health workers are asymptomatic carriers of pathogenic bacteria and thus represent a main source of contamination [29]. In a study conducted by [30], 91 workers from the 20 kitchens of all the public and private hospitals in Salvador, Brazil, were screened to identify their microflora profile using hand swabs, pharynx swabs and stool samples. Samples of the milk and infant formula delivered by these workers were also collected for microbiological analysis. The most common bacteria identified from hand swabs were *Staphylococcus* spp. (69.2%), *Bacillus* spp. (25.3%), *Pseudomonas* spp. (14.3%) and *Micrococcus* spp. (11.0%). Pathogenic bacteria such as *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *E. coli*, *Proteus mirabilis*, *Klebsiella oxytoca*, *Serratia* spp. were isolated from 20 (22.0%) hand swabs. No pathogenic microorganisms were detected in stool samples. The bacteria detected in milk and formula samples were mainly *Bacillus* spp. (30.8%), *Staphylococcus* spp. (13.2%) and *Pseudomonas* spp. (7.7%). Pathogenic bacteria such as *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia* spp., *Citrobacter freundii*, *Stenotrophomonas maltophilia* and *P. aeruginosa* were found in 17 (18.7%) samples of milk/formula. These data clearly suggested a link between bacterial contamination

of the milk/infant formula and the microflora profile of the health workers. *E. sakazakii* has been shown to be present at low number in prepared formula, while it has been detected in other types of food item, only prepared infant formula has been linked to outbreaks of disease in patients.

#### **Separation and Concentration of Microorganisms Present in Food:**

One major hindrance in detection of bacterial contaminants in food samples with high specificity and sensitivity is the low number of bacterial cells present in food items. This issue can be solved by separating and concentrating microorganisms in food samples to differentiate pathogens from other cells and use the appropriate concentration for enhancing the sensitivity level of the detection method. Several approaches such as antibody-based and various other physical- and chemical-based methods have been developed for the separation and concentration of microorganisms from food samples [31, 32]. For beverages and other liquid food, the concentration can be easily carried out by filtration or ultrafiltration methods [33, 34]. However, these methods cannot be employed for the selective isolation and concentration of the pathogens from solid foods. Magnetic separation techniques has shown lot of potential in diagnostic microbiology to specifically isolate microorganisms with high sensitivity from a complex mixture [35]. In this technique, superparamagnetic particles or polystyrene beads are coated with iron oxide and antibodies that bind specifically to pathogenic bacterial cells present in complex mixture [36]. The application of a magnetic field retains the particles with the attached cells and the rest of the organic and liquid material is easily removed by washing. Captured bacterial cells are plated to isolate and identify the major contaminants in food sample or tested using other assay. Immuno Magnetic separation (IMS) method along with other rapid and automated techniques had been used for the isolation and identification of pathogens such as *E. coli* O157:H7 [34, 37, 38]. However, IMS method does not yield a pure culture of the pathogen, hence other tests should be performed for more reliable detection of microorganisms in food samples [39] modified IMS technique by using samples volume 500 times larger than most common assays and by recirculating the sample during the capture phase in order to increase sensitivity and reduce detection times. In another strategy, bacteriophage tail-associated protein has been attached to paramagnetic beads instead of antibodies for capturing and isolating pathogenic bacteria

from the food samples. Bacteriophage-based capture kits are now available that can be used for rapid detection of pathogenic bacteria in various types of food samples [40].

**Solid-Phase Cytometry:** Solid-phase cytometry (SPC) is a technique that combines aspects of flow cytometry and epifluorescence microscopy. After filtration process, the retained pathogenic bacteria are fluorescently labeled with argon laser excitable dyes on the membrane filter and automatically counted by a laser scanning device. Each fluorescent spot can be observed with an epifluorescence microscope connected to a scanning device. Depending on the fluorogenic labels used, identification of the microorganisms can be accomplished in a very short period. SPC technique is more efficient if the number of bacteria present is high i.e  $10^3$  to  $10^4$  CFU/g. Although this method was originally recommended for the identification of microorganisms in liquid samples, it can also be employed for detection of pathogens in food samples, provided they can be specifically isolated. The efficiency of viable bacteria detection from food samples by IMS and then followed by SPC was evaluated using the pathogenic *E. coli* O157:H7 [41]. Within 5–7 h of enrichment, the IMS-SPC technique detected higher number of cells than were detected by plating. SPC has also been shown as an important technique to detect viable but nonculturable *Campylobacter jejuni* [42].

**PCR Based Detection Methods:** Cost effective and rapid diagnosis and understanding the mechanism for the causes of food-borne diseases are a major requirements for the food industry and public health. PCR has become a powerful tool for the microbiological diagnosis over the past decade [43]. A team of international experts from the European Committee was established for standardization of the protocols for the diagnosis of the food-borne illnesses by PCR technology [44, 12].

PCR-based approach for the detection of food-borne pathogens must fulfill various criteria such as high accuracy, improved sensitivity, cost-effectiveness, robustness and user-friendly protocols for its application and data analysis [44]. Real-time PCR technology has the capability to meet all these requirements by performing amplification and detection in a single-step process.

Conventional PCR method is based on amplification of the target gene(s), followed by separation of PCR fragments by gel electrophoresis, visualization and data analysis. The whole procedure can take few hours. The specificity can be subsequently confirmed by sequencing the amplified fragment. PCR can be more

sensitive and superior to culturing methods for detecting the main pathogens in food samples. Various other methods for detecting pathogens in food samples include Thermal and Denaturing Gradient Gel Electrophoresis (TGGE and DGGE), Single Stranded Conformation Polymorphism analysis (SSCP), Amplified Ribosomal DNA Restriction Analysis (ARDRA), Terminal Restriction Fragment (TRF) patterns and Amplified Length Heterogeneity analysis (ALH) [45].

Denaturing Gradient Gel Electrophoresis (DGGE) has become a useful technique for detecting microbial community in food after years of development [46]. It is capable of separating the PCR fragments of the similar size but with different DNA sequence. Separation of PCR fragments is based on the decreased mobility of a partially melted double stranded DNA molecule in polyacrylamide gels which contain a linear gradient of DNA denaturants or a linear temperature gradient. PCR fragments with different sequences would have different melting temperatures and thus are immobilized at different places in the gel [47]. With regards to dairy products, Coppola *et al.* (2001) used the PCR-DGGE with amplification of 16S V3 region to analyze the microbial community present in various types of Mozzarella cheese in Italy. This study demonstrated that DGGE analysis is more sensitive than 16S rDNA spacer polymorphism analysis and it is potentially more appropriate for analyzing the complex microbial diversity associated with dairy products [48].

The SSCP method is also capable of separating DNA fragments based on sequence composition. After the DNA from the samples is amplified by PCR, the amplicons are denatured into two single strand DNAs (ssDNA). The ssDNAs are then electrophoresed on nondenaturing polyacrylamide gel. The movement of the ssDNA in the polyacrylamide gel depends on its secondary structure which is further defined by the nucleotide sequence [49]. The SSCP method has been employed to study dairy products. Duthoit and colleagues [50] monitored the bacterial community dynamics in raw milk cheese by the 16S rRNA SSCP analysis and concluded that the SSCP was a powerful tool for monitoring the dynamics of global microbial population in food products. Delbes and colleagues [51] suggested that the SSCP could be used for monitoring microbial communities in raw milk and cheese by 16S rRNA gene based methodology [51,12].

In a RDRA, the total ribosomal DNA is extracted from samples and is amplified by PCR. This process yields a mixture of DNA fragments which represent all the species present in the samples. After PCR amplification, the

amplicons are further digested by restriction enzymes and analyzed by low resolution agarose gel electrophoresis [52]. The TRF method is based on the principal of ARDRA with some modifications. As with ARDRA, TRF analysis entails DNA extraction, PCR amplification and restriction enzyme digestion. However, one of the primers used for TRF is labeled with a fluorescent dye so that the size of the terminal restriction fragment (TRF) can be detected and the amount can be quantified when the fragments are analyzed in an automated DNA sequencer equipped with a laser detector [53]. TRF is now a common tool to study the microbial diversity of food samples. Rademaker *et al.* [54] performed the TRF analysis to study the surface microbial composition of bacterial smear-ripened Titlist cheese. Their study demonstrated that TRF analysis had the specificity to monitor the important members of the surface microflora [54].

#### **Metagenomics Approach to Analyze Food Microbiota:**

Metagenomics study using high-throughput sequencing can allow in-depth analysis of microbial diversity in food. The presence of microbial population in food samples can be evaluated by analyzing the microbiome using rRNA based high-throughput sequencing [46]. Analysis of fermented food using metagenomics approach can help in determining the bacterial population involved in fermentation as well as in detecting the microbial contaminants. Quigley *et al.* [55] analyzed about 60 Irish soft, semihard and hard cheeses by exploiting latest advancements in high-throughput sequencing to determine the microbial diversity based on cheese type, milk and various methodology for cheese production. The composition of cheese microbiota varied depending on the origin of milk, addition of different ingredients and also major differences were seen in microbial population of pasteurized and raw milk. It was observed that *lactobacillus* grew in large number in hard cheeses as compared to soft cheeses, suggesting an effect of cheese maturation on the growth of *lactobacillus*. Moreover, increase in salt concentration in cheeses led to the decrease in population of *leuconostoc* and *pseudomonas*. Similarly the addition of various ingredients such as herbs, spices or seaweed significantly altered the cheese microbiome [55]. High-throughput sequencing has also been used to monitor the changes in microbial population during long term storage of food. [56]. exploited the High-throughput sequencing technology to analyse the microbiota of beef stored for 40 days under different packaging conditions It was observed that *Brocothrix*

*thermosphacta* and *Pseudomonas sp.* were predominant in the first and second stages of air storage, respectively, on the other hand, *B. thermosphacta* and *Carnobacterium divergens* were predominantly present in the first and second stages of modified-atmosphere packaging storage, respectively. Moreover, *Lactobacillus* was detected during vacuum pack storage, while *C. divergens* was more predominant when meat was stored in nisin-activated anti-microbial packaging. Monitoring such changes in microbial population provides useful information which can be further used to select ideal storage conditions for food products in order to inhibit the growth of undesirable [56].

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