

Development of improved analytical methods for use in animal health and in foodborne disease surveillance for source attribution

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Summary

Considering the 'One Health' principles, the links between animal and human health are very strong. Both domestic and wild animals are sources of infectious agents that cause diseases in humans. Poor animal health may also indirectly affect human health, through reduced access to food. A large number of infectious diseases of animals, the transboundary animal diseases, spread rapidly across borders. Robust and accurate diagnostic assays are needed to detect the infectious agents rapidly and to limit their spread. A large arsenal of novel assays has been developed during the last three decades, with a tremendous impact on the detection of infectious agents. The new diagnostic methods are mostly laboratory-based and expensive, requiring sophisticated equipment and special skills. However, rapid and cheap field-based assays have also been developed. Herein, the authors give several examples of the development of novel assays, with special focus on the 'One Health' principles.

Keywords

Animal health – *Campylobacter* – Foodborne – Hepatitis E – Influenza – Molecular detection – Norovirus – Polymerase chain reaction – *Salmonella* – Transboundary animal disease – Zoonosis.

Introduction

The World Health Organization (WHO) states that, over the last decade, approximately 75% of the new diseases in humans were caused by pathogens from animals or animal products. Many of these diseases may spread extensively and become global problems (www.who.int/zoonoses/vph/en/). Thus, human and animal health are closely linked. Wild and domestic animals are important sources of emerging infectious diseases (EIDs) that may be transmitted to humans. These are a very significant burden on global economies and public health. Many are caused by viruses, especially RNA viruses that adapt easily to new hosts, and novel variants of bacteria (1, 2, 12, 19). An important

group of EIDs is the transboundary animal diseases (TADs), which are highly transmissible and spread rapidly across national borders, causing serious socioeconomic or public health consequences. Examples are foot and mouth disease (FMD) and African swine fever (ASF). Infections of animals may directly impact on human health by causing disease, or indirectly by depriving the human population of food. Thus, the fight against human diseases also includes improving animal health. Because several important animal diseases are TADs, rapid diagnostic measures, to reduce losses, spread and the consequences for human health, are needed. The occurrence and spread of TADs are facilitated by transported animals or migratory birds, which may rapidly spread infectious agents globally (5). Globalisation has led to an extensive and rapid distribution of animals

and animal products. This puts even higher demands on diagnostic services. The capability to rapidly diagnose and monitor the spread of diseases is of paramount importance for control of TADs. Early diagnosis is important in order to implement control measures as fast as possible and interrupt further spread. Sophisticated instruments and novel methods are developed continuously. Ideally, novel diagnostic tools have to be practical, robust and cheap, offering optimal sensitivity and specificity. Since the introduction of the polymerase chain reaction (PCR), a wide range of molecular diagnostic methods has been developed. The PCR assays offer very high sensitivity and specificity and are common in veterinary diagnostic laboratories today. The real-time PCR methods offer many advantages over conventional PCR assays, circumventing post-PCR analysis and opening reaction tubes, and give higher sensitivity. Additional advantages of real-time PCR are the rapidity, reduced risk of false-positive results, and the possibility of measuring virus load. Thus, a wide range of real-time PCR platforms offers powerful and affordable tools for diagnosis. It is important to note that real-time PCR is not a single technique, but has many variants. The TaqMan assays have a dominant position in diagnostics but chemistries such as SYBR-green and FRET-based assays, Molecular Beacons and Scorpion Primers, and the Primer-probe Energy Transfer systems are alternatives. For example, SYBR-green avoids the use of probes and is very economical. However, it is less specific, cannot differentiate between the amplified products and requires post-PCR melting curve analysis. Probe-based assays offer considerable advantages, including higher specificity and the possibility of detecting several agents simultaneously. The PCR-based diagnostic techniques are also becoming affordable under field conditions and in small clinics. Lightweight portable PCR instruments, which use batteries, can take the assays to the pen-side, offering a quick diagnosis 'on site' (7).

On-site diagnosis is further supported by the new isothermal amplification methods, such as loop-mediated isothermal amplification (LAMP), which run at a single temperature level on basic instruments and have results that can be read by the naked eye.

Further technical approaches, such as the padlock probes, proximity ligation assays and various liquid microarray readouts of PCR results, enable the simultaneous detection of a high number of different infectious agents on a single diagnostic platform (4, 7).

An economical alternative for on-site diagnosis involves lateral flow devices (LFDs), which have been developed for several agents, including FMD virus. They offer very quick results but with a lower sensitivity.

The so-called 'next generation' sequencing techniques are a completely new diagnostic concept. They can detect any nucleic acid in a sample. The results depend heavily on advanced bioinformatics, including comparative and advanced algorithms to analyse and understand the large volumes of nucleotide sequence data that are generated. These technologies are far too expensive for routine diagnostic purposes. However, with new technical solutions they may become affordable in the near future.

Below, the authors list several pathogens that are important, as TADs or foodborne infections, and briefly summarise the recent diagnostic achievements, with a special focus on the development of novel tools.

Highly pathogenic avian influenza

Avian influenza viruses (AIVs) constantly evolve and cause seasonal epidemics with a large socioeconomic impact and severe consequences for human and animal health. Infections by AIV subtypes are usually of low pathogenicity (LP), but introduction of low-pathogenicity avian influenza (LPAI) H5 or H7 subtypes to poultry may result in highly pathogenic (HP) variants, characterised by high mortality. Poultry infections by these subtypes are notifiable and the OIE *Terrestrial Animal Health Code* recommends trade only between areas without notifiable AIV (47). Thus, highly pathogenic avian influenza (HPAI) has affected poultry production and trade (1).

High pathogenicity of AIV is related to the sequence at the cleavage site of the haemagglutinin (HA) glycoprotein and very high diversity is observed in H5 and H7 AIVs. Current procedures for pathotyping involve sequencing (46).

Highly pathogenic avian influenza caused by H5N1 viruses is a serious problem in poultry in Asia and Africa, and is endemic in several countries (14). The circulation of H5N1 viruses among wild and domestic birds provides opportunities, during preparation of food or slaughtering of poultry, for human infections and more efficient transmission between humans (44). The issue of food safety regarding poultry and HPAI has been reviewed (5, 11). In general there is no transmission of AI to humans via consumption of poultry products. However, poultry meat may contain HPAI (17, 43), and both excretion of the virus in faeces and transmission to eggs occur (40). The surveillance of HPAI is complex, with LP and HP viruses co-circulating, asymptomatic or vaccinated birds shedding virus and wild birds transmitting the virus over large distances (15). Thus, there is a need for fast and inexpensive diagnostic methods for use under field conditions to improve food safety.

Recently, a novel strategy for pathotyping H5 and H7 AIVs was invented at the OIE Collaborating Centre for the Biotechnology-based Diagnosis of Infectious Diseases in Veterinary Medicine (Uppsala, Sweden) (30). The method provides a rapid, cheap and simple alternative to sequencing. Accordingly, it can be implemented for large-scale diagnosis and surveillance. This technique has been applied to pathotyping of other viruses, such as Newcastle disease virus (48). It is useful for rapid testing and surveillance in food safety and wild bird monitoring.

The method is a three-level semi-nested PCR system amplifying the cleavage site of HP and LP H5/H7 AIVs. The final step is constructed with plexor primers, and HP and LP primers are differently labelled. The assay is constructed to give a signal decrease for LP viruses and apparent signal increase for HP viruses. The assay concept was tested on 39 H5 clinical specimens from different tissues and feathers of experimentally and field-infected birds, and it correctly pathotyped all samples, even though the viral titres varied over five orders of magnitude. The strategy is an effective and robust method for notifiable avian influenza (NAI) pathotyping. Moreover, the method opens new opportunities for surveillance and diagnostics and it obtains the same pathotyping result as sequencing. This assay is suitable for portable PCR instruments and provides complete NAI diagnosis on site, near outbreaks. One particularly useful application for this assay may be in outbreaks where LP and HP variants are co-circulating (39).

Foodborne viruses, with special regard to noroviruses and hepatitis A viruses

Several viruses such as norovirus (NoV), hepatitis A virus (HAV), enteroviruses, rotaviruses and hepatitis E viruses (HEV) have been implicated in foodborne and/or waterborne outbreaks. In fact, all viruses that cause illness after consumption can potentially be spread via food. However, most reported cases are due to human NoV and HAV. These are considered the second most important human foodborne pathogens after *Salmonella* (24). Currently NoV is the leading cause of foodborne acute non-bacterial gastroenteritis (GE) worldwide, although HAV remains a major cause of acute viral hepatitis in developed countries (24).

Recent outbreaks of NoV and HAV have been associated with raw or poorly cooked seafood, fresh fruit, vegetables and berries, etc., but bivalve molluscan shellfish dominate

(29, 34, 37). Outbreaks linked to other foodstuffs have been documented (34).

The transmission routes for NoVs and HAV are similar. Both are very stable and highly contagious. Large numbers of viral particles are shed from infected persons (24).

Norovirus undergoes rapid genetic changes and new variants arise continually. Several variants co-circulate. Recently NoV has been reported in livestock, suggesting zoonotic transmission (45).

Hepatitis A virus causes acute viral hepatitis and is transmitted between humans through food or water. It causes a self-limiting disease with low mortality, but long convalescence.

Diagnosis of foodborne viral infections, with focus on noroviruses and hepatitis A viruses

A well-known problem of environmental virus detection is the low level of viral particles in the food, it is below the detection level, yet high enough to pose a health threat. Therefore, the highest diagnostic sensitivity is needed. In the last decade, nucleic acid amplification techniques, such as PCR, have been the basis for screening. There are numerous molecular diagnostic assays for detecting NoV and HAV in shellfish (9, 35). These viruses cannot be detected in tissue culture but electron microscopy and antigen detection are still used for NoV. Commercial immunoassays and real-time PCR are available for NoV in clinical samples, but are not suitable for food samples.

Currently, no standard method exists for the detection of NoVs and HAVs in food. The European Commission Regulation (EC) 2073/2005 lays down food safety criteria, but defines only bacteriological parameters. A working group of the European Committee for Standardization (CEN/TC 275/WG6/TAG4-viruses in foods) has developed standard methods for virus detection in foodstuffs (29).

Recent advances in foodborne virus detection

Nucleic acid amplification techniques, such as PCR, are widely used. However, during outbreaks tracing is required, which depends on detecting identical virus in clinical and food samples (29). In recent years, a variety of molecular approaches, such as microarray, multiplexing amplifications and nested amplification other than real-time PCR, have been developed. Several groups have developed microarrays for simultaneous detection, characterisation and separation of NoV and HAV (3, 10). However, the sensitivity of multiplex methods is often low (36).

Hepatitis E, a zoonotic disease

Hepatitis E (HE) is an important example of an endemic and emerging zoonotic viral disease of global importance. In South Asia and Africa, genotypes (gts) 1 and 2 are endemic in the human population, with outbreaks connected to poor sanitation. In Europe, Japan and China the disease is emerging, caused by gts 3 and 4, with relatively few but an increasing number of documented human cases. These gts are frequently detected in pig and wild boar populations and sometimes in other ungulates. Infections with gts 3 or 4 in humans are believed to come from pigs, but the infection route is unknown. Direct and indirect methods for detection of HEV have been developed. For indirect detection, several enzyme-linked immunosorbent assays (ELISAs) are available, but the inter-assay variability is considerable and they cannot differentiate gts. Direct detection is performed by molecular approaches (real-time PCR). Genotype and subgroup determination by sequencing facilitates tracing of the source of infection.

Salmonellosis

Salmonella, a Gram-negative microorganism, is widely dispersed in nature and often found in the intestines of animals. In humans, *Salmonella* can cause life-threatening illness, and it is the second most predominant bacterial cause of foodborne GE worldwide. Livestock harbour almost all serotypes (syn. serovars) of *Salmonella* in their gastrointestinal tract and faeces, and are the major source for contamination of soil, water and crops. Most human infections with *Salmonella* are, directly or indirectly, associated with contamination from beef, chicken, turkey, pork, eggs or milk. It is estimated that *Salmonella* causes 93.8 million cases of GE globally each year and nearly 80.3 million cases are foodborne (32). *Salmonella* is also an animal pathogen, and some serovars are more host-adapted.

The samples for detection of *Salmonella* include clinical, feed and environmental specimens. Generally, the diagnosis of salmonellosis is based on physiological and biochemical markers. Culture methods are the most widely used and appear to be a 'gold standard' owing to their high selectivity and sensitivity. The cultures are then subjected to biochemical and serological assays for definitive identification. Serotyping is essential for detection of strains. Over 2,500 serovars of *Salmonella* have been identified. The standard culture and serotyping methods are excessively time-consuming (five to seven days), leaving space for improvement and for the introduction of new techniques that are reliable, user-friendly, cost effective, and less laborious.

In order to minimise the time for detection and to avoid interference from food products and ingredients, debris,

background microorganisms, and lack of sensitivity, there has been great interest in improving the separation and concentration of samples before the application of the molecular detection techniques. Among various methods applied for this purpose, such as centrifugation, filtration and lectin-based biosorbents, the use of immunomagnetic separation (IMS) has received the highest appreciation and remains the most powerful tool for the separation and concentration of targeted pathogens, primarily owing to the reduced time required (one to two days) and high sensitivity. Moreover, the flexibility of IMS in conjunction with other assays, including electrochemiluminescence, ELISA, conductance microbiology, and PCR, makes it even more sensitive and unique.

Several serological methods, including rapid agglutination assays, ELISAs, and lateral flow immunoassays, are currently being used (22). Moreover, molecular methods are in use to characterise strains. Among these, pulsed-field gel electrophoresis (PFGE) is considered a gold standard. Methods for molecular detection such as ribotyping, insertion sequence typing, randomly amplified polymorphic DNA and amplified fragment length polymorphism (AFLP) have also been successfully applied. Multilocus sequence typing (MLST) appears to be highly valuable and practical for long-term epidemiological studies and phylogenetic analyses.

In the last few years, much attention has been given to the development of molecular approaches for the rapid detection of foodborne pathogens. The most successful tool has been real-time PCR, especially multiplex PCR (mPCR), which has resulted in the availability of a powerful and cost-effective tool for simultaneous detection of *Salmonella* Typhimurium, *Escherichia coli* and *Listeria* (22, 31, 38). Numerous commercial real-time PCRs are available for the detection of *Salmonella* in food and food ingredients, which target different genes of *Salmonella*. Some of these PCR assays are highly flexible, which allows rapid development of new assays targeting specific serovars from clinical samples. This flexibility is of special concern under the new regulations set out by the United States Food and Drug Administration for the poultry industry. To further enhance the efficacy and sensitivity, multiplex PCRs, in which several specific primer sets are combined, have been developed and commercialised. An mPCR that targets the *Prot6e* gene in the virulence plasmid of *S. Enteritidis* has successfully been evaluated for whole chicken carcass rinses and eggs (33). Similarly, an mPCR assay has been developed which can detect poultry-associated serotypes such as *S. Enteritidis*, *S. Gallinarum*, *S. Typhimurium* and *S. Kentucky* (38). In these and other PCR systems, the 16S rRNA gene is targeted for detection and differentiation. However, an mPCR has been reported recently in which the genes encoding either the phase 1 and 2 flagellar antigens, *fliC* and *fljB*, or unique serotype-specific loci were targeted. This assay can

rapidly screen 19 serotypes of *Salmonella*, and can detect multidrug resistance patterns for the ampicillin-amoxicillin, chloramphenicol-florfenicol, streptomycin-spectinomycin, sulfanomides, and tetracycline (ACSSuT) types (41). Based on this, it is plausible that mPCR assays have a future for the rapid and parallel differentiation of multiple species.

Following this objective, in 2003 Gilbert *et al.* developed an mPCR to rapidly and simultaneously detect *Salmonella* in combination with concurrently infecting pathogens such as *Campylobacter jejuni* and *E. coli* O157:H7 in a variety of raw and ready-to-eat food products (16). Later, in 2007, Kim *et al.* developed a novel mPCR for the simultaneous detection of five foodborne pathogenic bacteria: *E. coli* O157:H7, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Listeria monocytogenes* and *Salmonella* (23). Currently, advanced means of diagnosis and discrimination of *Salmonella* species are being implemented. Matrix-assisted laser desorption/ionisation–time of flight (MALDI-TOF)-based intact cell mass spectrometry (ICMS) has been applied for rapid discrimination of the clinically and epidemiologically important serovar S. Typhi and other non-S. Typhi serovars (25). However, it is noteworthy that most of the mPCRs, like many other molecular methods, have been developed and optimised on pure bacterial cultures and there have been only a few studies on the detection of pathogens in food products. Future studies of their applicability to clinical samples are required.

In conclusion, significant progress has been made to improve sample preparation techniques, isolation and detection of *Salmonella* in food products. However, all methods exhibit benefits and also limitations. Improvements are required in sample enrichment and preparation procedures and in the application of novel technologies such as biosensors, microarrays and nanotechnology for pathogen detection and disease surveillance.

Campylobacteriosis

Campylobacter spp. are zoonotic bacteria that cause acute GE in humans. The disease is one of the most commonly known bacterial enteric zoonoses worldwide, with more than 2.4 million estimated cases in the United States (www.cdc.gov) and over 200,000 confirmed cases annually in the European Union (13). *Campylobacter* spp. are Gram-negative, curved or spiral-shaped motile rods. The thermophilic species *C. jejuni* and *C. coli* are most frequently associated with human disease, accounting for more than 95% of cases (26).

Healthy wild and domestic animals are often carriers of the bacterium, and birds are considered the main reservoir for *C. jejuni*. The disease is mainly foodborne and important risk

factors for human infection are handling and consumption of undercooked poultry meat, contaminated drinking water, unpasteurised milk, contact with other animals and travel abroad (20).

Two subspecies of *Campylobacter fetus* are primary pathogens for sheep and cattle, but *C. fetus* is rarely associated with disease in humans. The focus here is on the thermophilic species *C. jejuni* and *C. coli* and the term *Campylobacter* will refer to them.

The prevalence of *Campylobacter* in the food chain is often monitored at slaughterhouse and at retail level. The samples usually consist of faeces, chicken caecal contents, carcasses, etc.

Traditionally, detection of *Campylobacter* involves culture of the sample, either directly or after pre-culture in selective enrichment broth. Incubation is done at 41.5°C in a microaerobic atmosphere. Another approach involves a filtration method with non-selective agar medium which allows isolation of antibiotic-sensitive strains of *Campylobacter*.

International standard culture-based protocols including confirmation by phenotyping and biochemical tests have been established for food samples. These methods are time-consuming, and take up to five to six days for a confirmed result.

In the last few decades, more rapid, non-culture-based methods have been developed for detection and identification, i.e. immunological and molecular assays. Simple latex agglutination tests for detection in faeces and confirmation of cultured *Campylobacter* have been described. The ELISA assays and lateral flow antibody-based immunoassays have been developed for detection mainly in food or human stool. The great advantages with these tests are their simplicity and speed. However, the performance and sensitivity vary. Results comparable to those of culture methods have been obtained after enrichment of samples or by combination with other techniques, e.g. ELISA-PCR (20).

Mass spectrometry is another strategy for identification of *Campylobacter*. This has lately become popular in clinical laboratories and MALDI-TOF has been described as a rapid tool for accurate identification at species level (8).

Since the 1990s numerous PCR-based assays, both conventional gel-based and real-time PCR tests, have been described. The PCR methodology provides rapid and reliable identification and differentiation of *Campylobacter* species (49). Detection in clinical samples and food by PCR may be hampered by inhibitory substances in the matrix

and the DNA extraction step is therefore very important. Real-time PCR assays for detection and quantification of *Campylobacter* in different matrices have been described (21, 28) and there are also commercial kits available for testing food. As for the immunoassays, many require an initial enrichment step in order to increase the sensitivity (20).

For epidemiological studies and tracing routes of infection, strains need to be characterised below species level. In the 1980s two serotyping schemes were developed. Although serotyping was considered a useful tool, problems with non-typable strains and poor availability of antisera have restricted its use and it has now been largely replaced by molecular typing methods. Several molecular techniques, basically of two categories, have been developed for the subtyping. In the first group are restriction-based methods producing DNA fragment banding patterns that are characteristic of the analysed strain, e.g. PFGE, PCR-restriction fragment length polymorphism (PCR-RFLP), and AFLP. PFGE has high discriminatory power and is useful for investigating outbreaks and small-scale epidemiology studies. It is considered a gold standard for subtyping *Campylobacter*. Two standardised protocols are accessible on the Internet: the PulseNet (www.pulsenetinternational.org/protocols) and Campynet (www.scribd.com/doc/38461463/PFGE-Protocol) protocols. In the second category are sequence-based techniques, e.g. MLST. Nucleotide polymorphisms in seven housekeeping genes constitute the basis for the MLST typing scheme. Sequence data are reproducible and exchangeable among laboratories

and are stored in a public database (<http://pubmlst.org/campylobacter>), and MLST typing has become widely used for epidemiological and population genetics studies of *Campylobacter*, especially for studying source attribution (42). A large number of genotyping methods have been developed and they all have their benefits and limitations (6). In the future, it is expected that more typing systems, based on whole genome sequences, will be developed and used. Rapid advances in sequencing technologies create large amounts of sequence data, which will provide great opportunities for this progress. However, it will require the expansion of bioinformatics platforms for management and analysis of the sequence data (18).

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Amélioration des méthodes analytiques visant à retracer la source des agents pathogènes dans le cadre de la surveillance des maladies animales et des toxi-infections alimentaires

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Résumé

Dans une perspective relevant du concept « Une seule santé », la santé animale et la santé humaine sont intimement liées. Nombre d'agents pathogènes qui affectent l'homme ont leur source chez les animaux domestiques ou sauvages. Une dégradation de la situation sanitaire des populations animales a des effets indirects sur la santé humaine, ne serait-ce qu'en raison de la pénurie alimentaire qui peut en résulter. De nombreuses maladies animales infectieuses se propagent rapidement d'un pays à l'autre : ce sont les maladies animales dites « transfrontalières ». Il est essentiel de disposer d'épreuves diagnostiques

fiables et robustes permettant de détecter rapidement les agents pathogènes et d'en contenir la propagation. En l'espace de trente ans, une très vaste panoplie d'épreuves nouvelles a été mise au point, transformant totalement notre capacité de détecter les agents pathogènes. Ces nouvelles méthodes diagnostiques sont généralement destinées aux laboratoires et nécessitent des équipements sophistiqués ainsi que des compétences spécialisées ; elles sont également très coûteuses. Toutefois, des tests de terrain rapides et bien plus abordables ont également été mis au point. Les auteurs examinent plusieurs de ces innovations en matière de diagnostic en mettant particulièrement l'accent sur les principes du concept « Une seule santé ».

Mots-clés

Amplification en chaîne par polymérase – *Campylobacter* – Détection moléculaire – Grippe – Hépatite E – Maladie animale transfrontalière – Maladie d'origine alimentaire – Norovirus – *Salmonella* – Santé animale – Zoonose.



Concepción de métodos analíticos perfeccionados aplicables a la sanidad animal y la vigilancia de enfermedades transmitidas por los alimentos con fines de atribución de focos

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Resumen

Atendiendo a los principios de “Una sola salud”, existe una relación muy estrecha entre la salud animal y la humana. Los animales, tanto domésticos como salvajes, son un foco de agentes infecciosos que provocan enfermedades en el hombre. Un deficiente estado de salud de los animales también puede afectar indirectamente a la salud humana porque reduce el acceso a los alimentos. Un gran número de enfermedades animales infecciosas, las transfronterizas, se propagan rápidamente a través de las fronteras nacionales. Para detectar sin tardanza agentes infecciosos y contener su propagación se precisan técnicas de diagnóstico robustas y exactas. En los últimos tres decenios se ha constituido un vasto arsenal de ensayos novedosos, que han ejercido una tremenda influencia en la detección de agentes infecciosos. Los nuevos métodos de diagnóstico se practican casi siempre en laboratorio, requieren instrumental sofisticado y conocimientos especializados y entrañan costos elevados. Pero también se han elaborado ensayos aplicables sobre el terreno, rápidos y baratos. Los autores exponen varios ejemplos relativos a la concepción de nuevos ensayos, haciendo especial hincapié en los principios de “Una sola salud”.

Palabras clave

Campylobacter – Detección molecular – Enfermedad animal transfronteriza – Hepatitis E – Influenza – Norovirus – Reacción en cadena de la polimerasa – *Salmonella* – Sanidad animal – Transmisión por vía alimentaria – Zoonosis.



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