SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 2 December 2015 PUBLISHED: 16 December 2015

doi:10.2903/j.efsa.2015.4330

Public health risks associated with Enteroaggregative *Escherichia coli* (EAEC) as a food-borne pathogen

EFSA Panel on Biological Hazards (BIOHAZ)

Abstract

Enteroaggregative *Escherichia coli* (EAEC) adhere to tissue culture cells in a stacked-brick pattern mediated by aggregative adherence fimbriae (AAF). EAEC strains often produce the heat-stable toxin EAST1, the *Shigella* enterotoxin (ShET1) and Haemolysin E. EAEC have been associated with cases of diarrhoea in travellers, children and immunocompromised patients and with urinary tract infections. Shiga toxin (Stx)-producing EAEC have been associated with Haemolytic Uraemic Syndrome and Haemorrhagic Colitis. EAEC are considered to be adapted to humans. In low-income countries animals may become exposed to EAEC from human waste. Food-related outbreaks of EAEC are frequently suggestive of cross-contamination by asympomatic food handlers. EAEC form biofilms, which has been linked to the severity of disease. The adhesion assay remains the most sensitive option for confirming isolates as EAEC. PCR provides accurate identification of EAEC and diagnosis of EAEC infections, but there is no consensus on a standard assay for the examination of foods. The protocol of the European Union Reference Laboratory for *E. coli* including Verocytotoxin-producing *E. coli* (EU RL VTEC) is considered a good candidate for the molecular detection of EAEC in food matrices by EU MSs. Whole genome sequencing (WGS) can provide data on the population structure of EAEC. Foodborne outbreaks of EAEC exhibiting antimicrobial resistance (AMR) have been reported but the origin of the resistance genes has not been fully established. Research needs include: (i) the development and validation of PCR-based methods for detection and quantification of EAEC in foods, and (ii) a standardised and validated multiplex approach to the identification of causal agents of diarrhoeal illnesses involving multiple pathogens. Surveillance needs include: (i) quantification of the possible involvement of EAEC strains in foods originating from low-income countries where sanitation is poor, and (ii) increased surveillance of foods associated with mixed pathogen outbreaks. When investigating foodborne outbreaks, testing for EAEC should be included as routine. Finally, WGS-based approaches for EAEC should be further explored.

Keywords: EAEC (Enteroaggregative *Escherichia coli*), diarrhoeal illness, virulence, detection, identification, foods

Requestor: EFSA

Question number: EFSA-Q-2014-00536

Correspondence: biohaz@efsa.europa.eu

Panel members: Ana Allende, Declan Bolton, Marianne Chemaly, Robert Davies, Pablo Salvador Fernández Escámez, Rosina Gironés, Lieve Herman, Kostas Koutsoumanis, Roland Lindqvist, Birgit Nørrung, Antonia Ricci, Lucy Robertson, Giuseppe Ru, Moez Sanaa, Marion Simmons, Panagiotis Skandamis, Emma Snary, Niko Speybroeck, Benno Ter Kuile, John Threlfall and Helene Wahlström.

Acknowledgements: The Panel wishes to thank the members of the Working Group on public health risks associated with Enteroaggregative *Escherichia coli* (EAEC) as a food-borne pathogen: Declan Bolton, Robert Davies, Gad Frankel, Claire Jenkins, Annemarie Käsbohrer, Stefano Morabito, Taina Niskanen, Flemming Scheutz and John Threlfall for the preparatory work on this scientific output and EFSA staff members: Maria Teresa da Silva Felicio, Pietro Stella and Emmanouil Chantzis for the support provided to this scientific output.

Suggested citation: EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2015. Scientific opinion on public health risks associated with Enteroaggregative *Escherichia coli* (EAEC) as a food-borne pathogen. EFSA Journal 2015;13(12):4330, 87 pp. doi:10.2903/j.efsa.2015.4330

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Summary

The European Food Safety Authority (EFSA) asked the Panel on Biological Hazards (BIOHAZ) to deliver a Scientific Opinion on the public health risks associated with Enteroaggregative *Escherichia coli* (EAEC) as a food-borne pathogen. In particular, EFSA requested the BIOHAZ Panel to: (i) provide an overview of interrelationships between different *E. coli* pathotypes together with their classification, pathogenicity mechanisms, and virulence determinants with particular reference to EAEC; (ii) assess the public health relevance of EAEC infections arising from the consumption of food; (iii) critically review phenotypic and genotypic methods for the detection, identification and characterisation of EAEC, with particular reference to putative isolates of EAEC from foods; (iv) critically review information on the nature, extent and public health relevance of antimicrobial resistance (AMR) in EAEC in relation to the food chain; and (v) identify research and surveillance needs to further understand EAEC as a foodborne pathogen.

To assist in the formulation of this Opinion, The European Centre for Disease Prevention and Control (ECDC) data on cases of human disease and EFSA data on food-borne outbreaks have been considered. A questionnaire on the occurrence of EAEC in humans has been circulated by ECDC to 30 countries in the ECDC Food and Waterborne Diseases and Zoonoses (FWD) network, and a questionnaire on the occurrence of EAEC in food, feed and animals was sent to the 31 countries in the Scientific Network for Zoonoses Monitoring Data (28 EU Member States (MSs), Iceland, Norway and Switzerland). A range of databases and search strings have been used to identify references and citations relevant to this Opinion, and data from a number of official EU publications and agencies, reports from the European Union Reference Laboratory for *E. coli* including Verocytotoxin-producing *E. coli* (VTEC) (= EU RL VTEC) and specific reports from MSs were utilised.

As data on EAEC are not collected and surveillance of EAEC in human diarrhoeal infections is not routinely undertaken by EU MSs, such information is therefore not available in the European Epidemiological Surveillance System (TESSy) database held by ECDC. Nevertheless, some relevant information can be derived from data for Shiga toxin (Stx)-producing *E. coli* (STEC), which are collected in TESSy. The EAEC plasmid-encoded regulator gene *aggR* and the chromosomally-mediated *aaiC* gene encoding secreted protein of EAEC were included in the specific set of optional reporting variables for STEC in the TESSy database after the 2011 Stx-producing EAEC O104:H4 outbreak. Data for the variable '*aggR*' are available and have been analysed from 2011 to 2013. Data on STEC were received from 24 MSs and two non-MSs. Five MSs reported a total of 44 *aggR*-positive isolates in two STEC serogroups: O104 and O127. The majority of cases in 2011–2013 were caused by *E. coli* of serogroup O104; there were also three cases of serogroup O127 (7%) and four cases (9%) were of unknown/not tested serotype (NT).

EFSA databases were examined for food-borne outbreaks reported in 2007–2013 that were associated with STEC and other pathogenic *E. coli*. Excluding water-borne outbreaks, 423 food-borne outbreaks were reported in those 6 years, of which 97 were classified as outbreaks with strong evidence (2010–2013) or as verified outbreaks (2007–2009). The causative agent was most commonly reported to be STEC O157, and occasionally Stx-producing EAEC O104:H4, STEC unspecified, Enterotoxigenic *E. coli* (ETEC) and Enteropathogenic *E. coli* (EPEC). Additional information on genes was inconsistently reported, and then only for concerned *eae* and *stx* genes. A wide variety of foods were reported to be the vehicle for the pathogens in these outbreaks, including foods of both animal and non-animal origin. Information in relation to food-borne outbreaks related to EAEC is therefore very limited, with the exception of reports relating to the 2011 EAEC O104:H4 outbreak. There are no reporting requirements in relation to the prevalence of EAEC in animals and foods.

In an introduction to the characteristics of the different *E. coli* pathotypes and their scientific literature, including previous EFSA Opinions, *E. coli* pathotypes have been designated using the standard four-letter abbreviations. In particular, when referring to Enteroaggregative *E. coli*, the term 'EAEC' has been used throughout this Opinion.

The Opinion provides general background information on the main characteristics of the different *E. coli* pathotypes, and particular focus has been placed on reviewing the pathogenicity mechanisms and virulence determinants related to EAEC. Of note is that there are EAEC strains which possess virulence characteristics of other pathotypes and such organisms may be highly virulent. An example

is the Stx-producing EAEC O104:H4 outbreak strain. Recent insights into the evolution and pathogenic potential of EAEC based on the use of genomics have been provided as an Appendix.

The Panel noted that gastroenteric *E. coli* strains are mainly divided into six pathotypes based on their pathogenicity profiles. These are: Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC) (including *Shigella* spp.), Enterotoxigenic *E. coli* (ETEC), Diffusely adherent *E. coli* (DAEC), STEC and EAEC. Further relevant pathotypes which may have exchanged genes with 'classic' gastroenteric pathotypes include adherent-invasive *E. coli* (AIEC) and extraintestinal pathogenic *E. coli* (ExPEC).

EAEC are characterised by their ability to adhere to tissue culture cells in a distinct stacked-brick pattern which is mediated by aggregative adherence fimbriae (AAF), of which there are several known isoforms. Expression of AAF is mediated by the plasmid-encoded transcriptional activator AggR; cell attachment is also mediated by the Toxigenic invasion locus A (Tia). EAEC strains often produce an enteroaggregative heat-stable toxin (EAST1) encoded by the plasmid-borne *astA* genes as well as *Shigella* enterotoxin (ShET1) and Haemolysin E (HlyE). Pathotype-determining factors for EAEC include *aggR, aaiC, aggA, aafA, agg3A, agg4A, agg5A,* AAF/I-V. EAEC lineages have evolved independently *via* multiple genetic events. The EAEC pan-genome is considered open and is still evolving by gene acquisition and diversification.

EAEC have been associated with travellers' diarrhoea, with acute diarrhoeal illness among children in both low-income and high-income regions, with severe diarrhoea in immunocompromised patients and with urinary tract infections (UTIs) in several countries worldwide, including EU MSs. The clinical manifestations of EAEC infection vary from individual to individual, depending upon the genetic composition of the host and of the strain. Infections with EAEC may be asymptomatic. Stx-producing EAEC have been associated with a range of symptoms such as haemolytic uraemic syndrome (HUS) and haemorrhagic colitis (HC), and have resulted in deaths in infected individuals. The emergence of mixed EAEC/STEC pathotype strains is likely to be an ongoing low frequency event and the occurrence of outbreaks probably relates primarily to opportunities for growth and dissemination of the organisms in foodstuffs or infected carriers. Strains of the EAEC pathotype are considered to be adapted to the human host. In low-income countries where sanitation is poor, animals may become exposed to EAEC originating from human waste. There is no evidence for animals being a reservoir of EAEC in EU MSs and outbreaks of EAEC associated with foods are frequently suggestive of contamination of the foods by asymptomatic food handlers. Multiple pathogen outbreaks in which a range of gastrointestinal (GI) pathogens, including EAEC are implicated, are being increasingly identified. EAEC strains are capable of forming biofilms and this property has been linked to the severity of human disease. Biofilms are also involved in survival of organisms on foods and in the environment.

Testing of food and faecal samples involves the detection of EAEC-associated traits in the matrix or in enrichment culture from such matrices, followed by isolation of the organism and confirmation of the presence of EAEC-associated genes or phenotypes. When reviewing phenotypic and genotypic methods for the detection, identification and characterisation of EAEC, with particular reference to putative isolates of EAEC from foods, the most widely-used option for confirming isolates as EAEC remains the adhesion assay, using monolayers of cultured epithelial cells. This approach is cumbersome, expensive and requires experienced personnel and is therefore for the most part confined to the reference laboratories. The widespread use of polymerase chain reaction (PCR) combined with the increased availability of information on the virulence gene asset of EAEC has led to the development of a number of gene-based assays. Such assays have been effectively used for the diagnosis of EAEC infections in humans but there is no consensus on a standard PCR assay to be used in the routine examination of foods for EAEC. Nevertheless, PCR-based methods can be used for the analysis of foods for the presence of EAEC-associated genes and the protocol developed by the EU RL VTEC based on the amplification of the *aggR* and *aaiC* genes is considered to be a good candidate for such investigations, and its ratification by EU MSs for this purpose has been recommended. Whole genome sequencing (WGS) can provide data on the population structure of EAEC but has not been used routinely for the identification of EAEC from either human infections or from foods. Further studies are required to assess the potential of WGS for such purposes.

Food-borne outbreaks of EAEC in which the causative strains have exhibited AMR have been reported, but the origin of the resistance genes in such strains has not been conclusively established.

A range of research and surveillance needs have been identified. Research needs include: the development of PCR-based methods for detection and quantification of EAEC in food items; a

standardised and validated multiplex approach to the identification of the causal agents of diarrhoeal illnesses that involve multiple pathogens; and controlled studies to fully quantify the survival characteristics of EAEC in wet and dry substrates under laboratory and natural conditions. Surveillance needs include quantification of the possible involvement in the EU of EAEC originating from parts of the world where pollution by human faecal waste is common *via* a survey of imported non-EU foods. For EU MSs sampling protocols for EAEC from food of non-animal origin should be developed and applied accordingly.

To enhance knowledge about the involvement of EAEC in food-related outbreaks, the development of a standardised and validated multiplex GI PCR approach for the detection of multiple GI pathogens, including EAEC, has been recommended. Such assays should provide a rapid, and cost-effective multi-pathogen approach for the detection of bacteria, viruses and parasites commonly associated with GI infection.

Revision of reporting requirements to encourage the provision of information by MSs on the number of tests for EAEC in different sample types and if so, the outcome of such tests under laboratory and natural conditions have been recommended, and when investigating food-borne outbreaks, testing for EAEC should be included as routine. Finally, WGS-based approaches for the identification of EAEC in foods should be further developed.

Table of contents

1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Pathogenic *Escherichia coli* are a diverse group of organisms divided into at least six groups based on the possession of one or more virulence characteristics. Such characteristics contribute to the ability of *E. coli* strains to cause disease in humans and have been used to define the groups. Because pathogenicity determinants are frequently carried on mobile genetic elements, such definitions are not rigid and organisms with characteristics of more than one group are increasingly being recognised as causes of severe human disease, and may have multiple reservoirs.

Enteroaggregative *E. coli* (EAEC, formerly known as 'EAggEC') are a heterogeneous group of *E. coli* first defined in 1987 (Nataro et al., 1987). Such organisms are primarily characterised by the presence of a specific pattern of 'stacked brick' aggregative adherence (AA) in human epithelial type 2 (HEp-2) cell assays. The diverse nature of EAEC is such that not all strains are able to cause disease. Conversely, other EAEC possess additional virulence determinants which have been associated with the ability to cause diarrhoea and other symptoms, and which might be life-threatening in vulnerable patients.

1.1.1. Epidemiology of EAEC

Early studies highlighted the association between EAEC and persistent diarrhoea in children, predominantly in low-income countries (Nataro and Kaper, 1998). More recently, EAEC have been identified as an important cause of acute diarrhoea in children and adults in a wider range of countries, including parts of Europe and in travellers returning from countries outside the European Union (EU).

Relatively little is known about the burden of EAEC in infectious intestinal disease (IID) in the EU and the reservoir(s) and pathways of infection, including transmission *via* foods. In a UK population-based study of IID in the Community in 1993–1996, EAEC were the most commonly isolated enterovirulent *E. coli* in cases presenting to a doctor (FSA, 2000). In a second IID study in the UK in 2008–2009, EAEC were isolated from more than 1.9% of cases in the population and 1.4% of cases presenting to a doctor (Tam et al., 2012a; Tam et al., 2012b). In a recent European study of Extended Spectrum Beta (β)-lactamase (ESBL)-producing *E. coli* from cases of human infection, food-producing animals and food in three European countries, screening of 359 ESBL-producing *E. coli* detected 15 (4.1%) enteropathogenic *E. coli* (EPEC), 11 (3.1%) EAEC and a single (0.2%) Shiga toxin (Stx)-producing *E. coli* (STEC) (0.2%) (Chattaway et al., 2014a).

1.1.2. Methods of identification of EAEC

Several methods have been used for the identification of EAEC from cases of diarrhoea. These include a PCR assay, clump formation test (visible bacterial aggregation at the surface of liquid culture medium), glass slide adherence assay, and the conventional HEp-2 cell adherence assay. As yet, no definitive method for the identification of EAEC from foods has been recommended.

1.1.3. Foodborne transmission of EAEC

There is evidence in the literature of foodborne transmission of EAEC, mostly through documented outbreaks and case-control studies (see Section 3.2.2). Of particular note is the major outbreak of Stx-producing EAEC serotype O104:H4, first identified in northern Germany in May 2011 and epidemiologically associated with the consumption of fenugreek sprouts (Buchholz et al., 2011; EFSA, 2011b). This outbreak resulted in over 4,000 confirmed cases of infection, with 54 deaths reported in 14 EU countries, the USA and Canada (Buchholz et al., 2011; Karch et al., 2012). The causative strain included features typical of EAEC, together with the capacity to produce Stx 2a (Frank et al., 2011), and also possessed a distinct set of additional virulence and antimicrobial resistance (AMR) genes (Rasko et al., 2011; Scheutz et al., 2011). Although not considered important in this outbreak, as antibiotic resistance may impinge on treatment in vulnerable patients, the nature, extent and relevance to public health of antimicrobial resistance in EAEC in relation to the food chain requires critical evaluation (Boisen et al., 2015).

1.1.4. Terms of reference

The European Food Safety Authority (EFSA) will address this self-task mandate through the elaboration of a scientific Opinion to be adopted by the Biological Hazards (BIOHAZ) Panel. The terms of reference (ToRs) are:

1. Provide an overview of interrelationships between different *Escherichia coli* pathotypes together with their classification, pathogenicity mechanisms, and virulence determinants with particular reference to enteroaggregative *E. coli* (EAEC).

2. Assess the public health relevance of EAEC infections arising from the consumption of food.

3. Critically review phenotypic and genotypic methods for the detection, identification and characterisation of EAEC, with particular reference to putative isolates of EAEC from foods.

4. Critically review information on the nature, extent and public health relevance of antimicrobial resistance in EAEC in relation to the food chain.

5. Identify research and surveillance needs to further understand EAEC as a foodborne pathogen.

1.2. Interpretation of the Terms of Reference

In light of the available scientific knowledge on EAEC, the above ToRs have been further discussed within the EFSA BIOHAZ Panel and its *ad hoc* Working Group (WG). Each individual ToR is further examined below and its interpretation in the framework of this Scientific Opinion is presented.

1.2.1. Term of Reference 1

When considering ToR 1 of the mandate, a brief introduction on the characteristics of the different *Escherichia coli* pathotypes and their interrelationships will be provided in this Opinion, referring to and updating the information included in a recent EFSA Scientific Opinion on STEC[1] seropathotype and scientific criteria regarding pathogenicity assessment (EFSA BIOHAZ Panel, 2013). For clarity purposes, and in accordance with the vast majority of the publications available in the scientific literature, including previous EFSA Opinions, *E. coli* pathotypes will be designated using the standard four-letters abbreviations. In particular, when referring to Enteroaggregative *E. coli*, the term 'EAEC' is used throughout this Opinion. This has to be intended as a synonym of the term 'EAggEC', now more rarely used within the scientific community. It should be noted that there are EAEC strains with characteristics from other pathotypes. An example of this is the Stx-producing EAEC O104:H4 (see Section 1.1.3 above).

While providing general background information on the main characteristics of the different *E. coli* pathotypes, particular focus will be placed on reviewing the pathogenicity mechanisms and virulence determinants related to EAEC. Recent insights into the evolution and pathogenic potential of EAEC based on the use of genomics have been provided as an Appendix to the Opinion (Appendix F).

1.2.2. Term of Reference 2

As indicated in Section 1.1, EAEC are a heterogeneous group of *E. coli.* According to the epidemiological evidence available, different sources have been recognised as the origin of EAEC responsible for human disease, including food. Information on the transmission pathways is still limited. Therefore, when answering ToR 2, the Opinion will review and discuss the public health relevance of EAEC in general, while giving particular attention to the assessment of the occurrence and severity of infection with EAEC originating from handling, preparation and consumption of food. In the context of this Opinion, '*handling and preparation'* should be interpreted as '*handling and preparation of food that occurs immediately prior to consumption*'.

[1] Shiga toxin-producing *Escherichia coli* (STEC) is also known as verotoxigenic *E. coli*, verocytotoxigenic *E. coli*, verotoxin producing *E. coli* and verocytotoxin-producing *E. coli* (VTEC).

Since the level of risk posed by a pathogen in a certain food depends on a number of factors, including the concentration of the pathogen in the ingested food, factors contributing to the survival and growth of EAEC in food will be reviewed and discussed.

1.2.3. Term of Reference 3

When considering ToR 3, a review and an assessment of the available laboratory methods to detect, identify and characterise EAEC will be performed. Advantages and disadvantages of the different methods will be highlighted, with a view to identify the best available diagnostic options. Since no definitive method has been recommended for the detection and identification of EAEC in food, the Opinion will focus on the methods that would be appropriate for use in food matrices, including consideration of suitable EAEC molecular markers.

1.2.4. Term of Reference 4

The information in relation to antimicrobial resistance (AMR) in EAEC in the food chain is scarce. Therefore the Opinion will consider the ToR from a wider perspective, with the aim of assessing the public health relevance of AMR in EAEC in general. In this respect the occurrence of AMR in EAEC will also be compared with AMR in other pathogenic *E. coli* groups, particularly STEC, which are known to have a food animal reservoir. The possibility of treatment failure in cases where antimicrobial therapy is indicated will be considered.

1.2.5. Term of Reference 5

When answering to ToR 5, the Opinion will provide recommendations in relation to research and surveillance needs that would help in better understanding the role of EAEC as a potential food-borne pathogen. The Opinion will consider the need to monitor EAEC in foods of both animal and non-animal origin, and in food-producing animals, aiming at acquiring better knowledge on the distribution of EAEC and on the potential sources of EAEC from cases of human infections.

2. Data and methodologies

2.1. Data

2.1.1. ECDC data on cases of human disease

Specific data on EAEC are not collected and are therefore not available in the European Epidemiological Surveillance System (TESSy) database held by the European Centre for Disease Prevention and Control (ECDC) database. As such, there are no ECDC data on EAEC that are non-STEC. Nevertheless, some relevant information can be derived from data for STEC, which are collected in TESSy, as defined in the EU case definition (Commission Implementing Decision 2012/506/EU[2]). The EAEC plasmid-encoded regulator gene *aggR* and the chromosomally-mediated *aaiC* gene encoding secreted protein of EAEC were included in the specific set of optional reporting variables for STEC in the TESSy database after the 2011 Stx-producing EAEC O104:H4 outbreak (see above). Data for the variable '*aggR*' are available and have been analysed from 2011 to 2013.

Data on STEC were received from 24 Member States (MSs) and 2 non-MSs. Five MSs reported a total of 44 *aggR*-positive isolates in two STEC serogroups: O104 and O127 (in addition to four cases of unknown serotype – 'NT'). The majority of cases (37 out of 44 cases) in 2011–2013 were caused by *E. coli* of serogroup O104; there were also 3 out of 44 cases of serogroup O127 and 4 out of 44 cases were NT. Serogroup O104 cases were mostly (35 out of 44 cases) reported in 2011. The reported 35 *aggR*-positive O104 cases represented 3% of all STEC O104 cases (1,066 cases) reported in TESSy in 2011. From 2012 to 2013, two additional *aggR*-positive serogroup O104 cases were reported. Germany did not report any detailed information on isolates related to the 2011 outbreak; 25 cases out of all 35 STEC O104 cases in 2011 were reported by one country (Denmark).

[2] Commission Implementing Decision 2012/506/EC of 8 August 2012 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council. OJ L 262, 27.9.2012, p. 1–57.

One country reported other *aggR*-positive isolates (serogroup O127 and NT) in 2011-2013. The typical characteristics of such *aggR*-positive isolates in both serogroups were: all were Shiga toxin-encoding gene (*stx1)*-negative, *stx2*-positive, negative for *eae,* the intimin-encoding gene of Stx-producing *E. coli* (except one NT *eae*-positive) and *aaiC*-positive (except seven *E. coli* O104 isolates of unknown status, i.e. information on the *aaic* gene was not available). The majority of the cases were hospitalised, 15 out of 17 cases with known data. The most common symptom was bloody diarrhoea (72%). There were no deaths among the *aggR*-positive cases where the outcome of disease was reported (41 cases; 93%) nor was there any information about the food source for any of the reported *aggR*-positive cases.

2.1.2. EFSA data on food-borne outbreaks

Data in relation to zoonoses, zoonotic agents and food-borne outbreaks are collected annually by EU MSs, as prescribed by Directive 2003/99/EC.[3] Those data are elaborated and published yearly by EFSA and ECDC in the EU Summary Report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks (EFSA and ECDC, 2013). EFSA's Zoonoses database represents the best current source in the EU to link cases, pathogens and food vehicles. These outbreak data are reliant on reporting practices, which can be incomplete, vary between MSs, are greatly influenced by rare events occurring during the monitoring period, or be biased due to the preferential investigation of types of foods perceived as posing higher risk or caused by hazards which are easier to identify. Nevertheless, this zoonosis reporting represents the most comprehensive set of data in the EU.

A search among the published data available from the Zoonoses database (years 2007–2013) has been performed in order to collect information on food-borne outbreaks associated with pathogenic *E. coli* in general, and EAEC in particular.

As of 2007, harmonised reporting specifications were applied by all MSs distinguishing outbreaks where the link between human cases and implicated foodstuffs was established by laboratory analysis or epidemiological evidence ('verified outbreaks') and other outbreaks ('possible outbreaks'). Detailed information was reported only for the former. Starting from 2010, new revised specifications were applied, and outbreaks are since then classified as having 'strong evidence' or 'weak evidence' of the implication of a suspected food vehicle, on the basis of the strength or epidemiological and microbiological evidence available.

EFSA databases were examined for food-borne outbreaks reported in 2007–2013 and associated with STEC and other pathogenic *E. coli*. Excluding water-borne outbreaks, 423 food-borne outbreaks were reported in those 6 years, of which 97 were classified as outbreaks with strong evidence (2010–2013) or as verified outbreaks (2007–2009). The causative agent identified was mainly reported to be STEC O157, and occasionally Stx-producing EAEC O104:H4 (2011 German outbreak and related secondary outbreaks), STEC unspecified, Enterotoxigenic *E. coli* (ETEC) and Enteropathogenic *E. coli* (EPEC). Additional information on genes identified was inconsistently reported, and only concerned *eae* and *stx* genes.

A wide variety of foods were reported to be the vehicle of the pathogens in these outbreaks, including foods of animal origin (e.g. bovine meat, porcine meat, broiler meat, milk, dairy products, fish) and of non-animal origin (vegetables, salads, sprouts, herbs, and spices). Information in relation to food-borne outbreaks related to EAEC is therefore almost absent, with the exception of the 2011 EAEC O104:H4 outbreak (see Section 1.1.3 above).

2.1.3. EFSA data on animal/food prevalence

As mentioned above (Section 2.1.2), Directive 2003/99/EC obliges EU MSs to monitor and report data in relation to prevalence of zoonotic agents in animals, foods and humans. Collection of such data is mandatory for some pathogens only, including STEC, but excluding other *E. coli* pathotypes, for which few data have been therefore reported during 2007–2013. It should be noted that monitoring and surveillance schemes for most zoonotic agents reported *via* EFSA's Zoonoses web-based application are not harmonised between MSs, and findings must therefore be interpreted with care. The data

[3] Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. OJ L 325, 12.12.2003, p. 31–40.

presented may not necessarily derive from sampling plans that are statistically designed, and may not accurately represent the national situation across the EU regarding zoonoses. Moreover, when interpreting the *E. coli* data it is important to note that information from different investigations are not necessarily directly comparable between MSs owing to differences in sampling strategies and the analytical methods applied.

There are no reporting requirements in relation to the prevalence of EAEC in animals and foods. Such data are therefore not available in EFSA zoonoses database.

Prevalence data have been sporadically reported for the following categories: non-pathogenic *E. coli*, unspecified pathogenic *E. coli*, STEC, enteroinvasive *E. coli* (EIEC), EPEC, and enterotoxigenic *E .coli* (ETEC). More detailed information on the serotype is only reported for STEC isolates. Information on genes was seldom reported, and then only concerns *eae* and s*tx* genes.

2.1.4. Questionnaire on EAEC in food, feed and animals

In an attempt to ascertain information on the occurrence of EAEC in food, feed and animals in the EU, a questionnaire (Appendix A) was sent to the 31 countries in the Scientific Network for Zoonoses Monitoring Data (28 EU MSs, Iceland, Norway and Switzerland). The questionnaire requested: details of methods in use (if any) for the detection of EAEC in food/feed/animal samples; if EAEC were searched for in food/feed/animal samples; details of the food matrices sampled (if any); if any additional characterisation was performed on EAEC isolates in food/feed/animals (e.g. serotype, AMR profile etc.); data on the occurrence of EAEC in food/feed animals published in official monitoring reports and if so, details of such reports and how they may be accessed; and finally, were responders aware of any additional EAEC data other than the official monitoring (e.g. research activities) in their country.

Replies were received from 23 EU/EEA countries, a response rate of 74.1% (Appendix B). These indicated that 20/23 (86.9%) countries have implemented a method for the detection of EAEC in food/feed/animal samples. No country has an accredited method for the detection of EAEC in food/feed/animal samples, although in one country the National Reference Laboratory (NRL) foresees the accreditation of the method of the EU Reference Laboratory (EU RL) for *Escherichia coli* including VTEC (= EU RL VTEC) in 2016. The number of official food samples tested ranged from 601 in 2013 to 3,099 in 2014; of these, no EAEC-positive samples were detected. In respect of other food samples tested, 21 were screened in 2013 and 190 in 2014, of which for each year 2 were EAEC-positive. No data on the occurrence of EAEC in food/feed animals were published in official monitoring reports in any country. Four countries were involved in research activities involving putative EAEC from food animals and food (Appendix B).

2.1.5. Questionnaire on EAEC in humans

To obtain data on cases of EAEC infections a parallel questionnaire was circulated to 30 countries (28 EU MSs, Iceland and Norway). in the ECDC Food and Waterborne Diseases and Zoonoses (FWD) network (Appendix C). This questionnaire asked: whether MSs have implemented a method for the detection of EAEC in stool samples; whether diarrhoeal samples were searched for EAEC and if so, the methods used; details of the numbers of samples studied, EAEC positive findings and EAEC cases in the respective countries in 2012, 2013 and 2014; the most common EAEC serogroups; the number of EAEC outbreaks in the respective countries in 2012, 2013 and 2014; whether a food vehicle had been associated with any of the outbreaks; data on the occurrence of EAEC infections published in official monitoring reports in the respective countries; and if so, information about such reports and how they may be accessed; and finally, details of examples of cases of infection other than those regarded as diarrhoeagenic in the respective countries over the last 10 years (e.g. UTIs), where EAEC may be involved.

Replies were received from 22 EU/EEA countries, a response rate of 73% (Appendix D). Nineteen (86%) of these countries have a method available for detection of EAEC from stool samples. Of note are comments that for one country the data are from the National Reference Laboratory (NRL) but are not representative since EAEC is not under surveillance and for another country, that EAEC is not under national laboratory-based surveillance and strains or samples are not sent to the NRL. In this country results are from one clinical microbiology district hospital laboratory performing a PCR assay

for detection of several pathogens, including EAEC, from stool samples from patients with diarrhoea. For a further country *aggR* is screened for in all *E. coli* isolates received at their NRL but none of the clinical microbiological laboratories routinely screen for EAEC; *aaiC* is screened for in all *eae*-negative STEC from patients with severe clinical symptoms; such tests are undertaken at the NRL. In one country the NRL implemented a method for the detection of *aggR* gene since the 2011 O104:H4 outbreak. This method is only used for the detection of the gene in STEC strains that are negative for *eae* gene. The resultant data therefore reflect only the number of strains that have *stx* genes and *aggR* virulence factors.

Seventeen of the 19 (90%) countries screened for EAEC used PCR, with several different genes amplified; two countries (20%) relied only on bacterial culture. There was considerable variation in the numbers of samples tested from 2012 through to 2014, ranging from approximately 13,000 in 2012, 4,300 in 2013, and 6,800 in 2014; in these years between 2.9% and 7.0% were EAEC-positive. Eleven countries reported on the numbers of EAEC cases identified, with figures ranging from 420 in 2012 to 961 in 2014. Five countries reported on serogroups or serotypes and over 20 different serotypes were identified. Of note is that not all countries report cases every year, and from 2012 through to 2014 only five countries reported cases annually.

The number of EAEC outbreaks ranged from 6 in 2012 to 24 in 2014; these reports came only from two countries. Two countries reported on food vehicles associated with the EAEC outbreaks. These were unpasteurised cheese made from ovine milk (one country, 2006) (Scavia et al., 2008) and curry leaves (one country, 2013). Only one country (Germany) published data about the annual number of EAEC infections in official monitoring reports.[4] None of the responding countries provided information on putative EAEC involvement in cases of infection other than those regarded as diarrhoeagenic over the last 10 years. Finally, two countries mentioned EAEC infections in returning travellers, and one of these countries made the observation that the majority of the positive results were from a cohort study indicating that the carrier state may apply to 4% of people and is especially related to travellers.

2.2. Methodologies

2.2.1. Databases and search strings

A range of databases and search strings have been used to identify references and citations relevant to this Opinion. These are summarised in Table 1 below.

Table 1: Databases, search strings and time spans of the literature searches performed for different sections of the Opinion

Subject area	Search database[(a)] (Search fields)	Search strings	Time span
Methods of detection, identification and characterisation of EAEC	PubMed (title/abstract) WoS (topic)	• (Enteroaggregative OR *E. coli* OR EAEC) AND • (detection OR identification OR characterisation) AND • virulence genes	All years
Occurrence of EAEC in food	Science Direct (abstract, title, keywords) PubMed (title/abstract)	• (EAEC OR eagg OR enteroaggregative OR EAggEC) AND • (Food OR Meat OR Beef OR Lamb OR Pork OR Poultry OR Dairy OR Cheese OR Fruit Or Cereals OR Water)	All years

[4] Robert Koch-Institut: Infektionsepidemiologisches Jahrbuch für 2014, Berlin, 2015.

Subject area	Search database[a] (Search fields)	Search strings	Time span
Occurrence of EAEC in animals	Science Direct (abstract, title, keywords)	• (EAEC OR eagg OR enteroaggregative OR EaggEC) AND • (Animals OR Cattle OR Bovine OR Sheep OR Ovine OR Pigs OR Porcine OR Poultry OR Horses)	All years
Occurrence of EAEC in animals[b]	WoS (topic)	• (EAEC OR eagg OR enteroaggregative OR EaggEC)	All years
Occurrence of EAEC in environment[b]	WoS (topic)	• EAEC OR eagg OR enteroaggregative	All years
Factors affecting the survival of EAEC[b]	WoS (topic)	• EAEC OR eagg OR enteroaggregative	All years
Biofilm formation by EAEC[b]	WoS (topic)	• EAEC OR eagg OR enteroaggregative	All years
Clinical symptoms and severity of illness	PubMed (title/abstract)	• (EAEC OR eagg OR Enteroaggregative) AND • (disease OR symptoms)	All years
Occurrence of EAEC in urinary tract infections (UTIs)	PubMed (title/abstract) Science Direct (abstract, title, keywords)	• (EAEC OR eagg, OR enteroaggregative OR EaggEC) AND • UTI AND • (UPEC OR Urinary)	All years
Antimicrobial resistance in EAEC	WoS (topic), PubMed (title/abstract) Science Direct (abstract, title, keywords)	• (E. coli OR EAEC OR VTEC OR EAggEC) AND • (toxin OR antimicrobial resistance OR antibiotic resistance)	All years followed by Monthly searches from start of opinion and ongoing
AMR comparison with other pathotypes	PubMed (title/abstract)	• STEC OR VTEC AND • (antimicrobial resistance OR antibiotic resistance)	All years and on going
Type VI secretion system	PubMed (title/abstract)	• EAEC AND • T6SS, AND • AggR	All years
Virulence factors	PubMed (title/abstract)	• EAEC AND • (Virulence OR • Pathogenesis)	All years
aggR	PubMed (title/abstract)	• EAEC AND • aggR	All years
aaiC	PubMed (title/abstract)	• EAEC AND • aaiC	All years
Biofilm	PubMed (title/abstract)	• EAEC AND • Biofilm	All years
Colonization	PubMed (title/abstract)	• EAEC AND • colonization	All years
Infection	PubMed (title/abstract)	• EAEC AND • infection	All years

EAEC/EAggEC: Enteroaggregative *E. coli*; STEC: Shiga-toxin producing *E. coli*; VTEC: verocytotoxin-producing *E. coli.*

(a): Key: WoS, Web of Science; PubMed, US Library of Medicine, National Institutes of Health.

(b): The retrieved relevant references were manually screened and included in the relevant sections of the Opinion.

An example of the reference searches utilised is that of the use of Web of Science (WoS) with the following search string: 'EAEC OR eagg OR enteroaggregative' on 11 November 2014. This retrieved 498 relevant references which were transferred into Endnote to form a list including abstracts. All of these were manually screened and references mentioning animals OR biofilms OR environment were retrieved and included in the relevant sections of the Opinion. In addition to this, each month two searches with the keywords string '(*E. coli* OR EAEC OR VTEC OR EAggEC) AND (toxin OR antimicrobial resistance OR antibiotic resistance)' were run in Web of Science (WoS). Two additional references were identified after the initial search concerning EAEC and antimicrobial resistance, and were considered for incorporation in the text. A further example is the use of PubMed in March 2015 for citations relevant to clinical symptoms and disease severity, utilising the following search string: '(EAEC OR eagg OR Enteroaggregative) AND (disease OR symptoms)'. This retrieved 105 citations, of which 47 were considered relevant to the section. After discussion, a further five citations suggested by members of the WG were included in the text.

2.2.2. Other methods for data gathering and analysis (data mining)

Relevant data from a number of official EU publications and agencies, reports from the EU RL VTEC and specific reports from MSs were utilised as appropriate. Such reports are shown in Appendix E.

Finally, considerable use was made of ongoing citation input by WG members and information about relevant publications provided by past and present members of the EFSA Biological Hazards (BIOHAZ) Panel.

3. Assessment

The information presented below is a summary of current[5] knowledge on the properties of pathogenic *E. coli* that have been identified in cases of in diarrhoeal disease in humans, with particular respect to EAEC, and the virulence determinants therein. This summary should not be regarded as definitive, as many of the virulence factors are carried on mobile bacterial genetic elements or by bacteriophages, and new strains with various virulence gene combinations are constantly emerging.

3.1. Interrelationships between *Escherichia coli* pathotypes (ToR 1)

3.1.1. Introduction

Escherichia coli, discovered in 1885, is one of the most broadly studied bacterial species. While *E. coli* is part of the endogenous intestinal microbiota (Kaper et al., 2004), some strains are pathogenic and can cause disease in humans and animals (Gyles, 1994). *E. coli* strains can be categorized by their serogroup, e.g., *E. coli* O157 where O refers to the lipopolysaccharide (LPS) O-antigen or serotype e.g. *E. coli* O157:H7 where H refers to the flagellar antigen. Importantly, *E. coli* strains are easy to grow, handle and genetically manipulate in the laboratory, and can naturally acquire mobile genetic elements.

Pathogenic *E. coli* strains can infect diverse sites and cause a range of symptoms ranging from mild diarrhoea to life-threatening conditions (Nataro and Kaper, 1998; Kaper et al., 2004; Clements et al., 2012). In particular, *E. coli* can cause enteric/diarrhoeagenic or extra-intestinal (ExPEC) infections in humans. ExPEC infections are primarily urinary tract (caused by uropathogenic *E. coli* (UPEC)) and sepsis/meningitis (caused by neonatal meningitis *E. coli* (NMEC)).

Diarrhoeagenic *E. coli* strains are conventionally divided into six pathotypes based on their pathogenicity profiles (virulence factors, clinical disease and phylogenetic profile) (reviewed by Tozzoli and Scheutz (2014)): EPEC, EIEC (including *Shigella* spp.), ETEC, Diffusely Adherent *E. coli* (DAEC), STEC, and EAEC (Tozzoli and Scheutz, 2014). Additional pathotypes include Adherent Invasive *E. coli* (AIEC), which is thought to be associated with Crohn's disease (CD) but does not cause diarrhoeagenic infection (Martinez-Medina and Garcia-Gil, 2014). Importantly, each pathotype contains many serotypes (e.g. 117 ETEC serotypes have been identified) (Tozzoli and Scheutz, 2014) and as some pathotypes can belong to more than one serotype, serotyping may not provide definitive identification of pathotypes.

[5] Up to, and including, data published by 3 December 2015.

The main factors (molecular and/or phenotypic) defining each of these pathotypes is listed in Table 2. As *E. coli* can exchange and combine some of these factors, it is challenging to unambiguously define all the different *E. coli* pathotypes.

Human infections with pathogenic *E. coli* occur *via* ingestion of food products (e.g. undercooked meat, contaminated fresh produce etc.), or drinking water contaminated with animal or human waste, or *via* the faecal-oral route (EFSA BIOHAZ Panel, 2013). Due to variability in clinical presentations and in the extent of detection and reporting, it is somewhat difficult to determine the exact figures of the incidences of enteric *E. coli* infections worldwide. In low-income countries EAEC, ETEC and EPEC appear to be major causes of infantile, potentially fatal, diarrhoea (Kotloff et al., 2013).

3.1.2. Diarrhoeagenic *E. coli* pathotypes

Enteropathogenic *E. coli* (EPEC)

EPEC remains an important cause of infant diarrhoea in low-income countries, although studies of the epidemiology of EPEC indicated that EPEC is still an important cause of infantile diarrhoea in England (Sakkejha et al., 2013) and Denmark (Scheutz et al., 2014). EPEC strains carry the *eae* gene encoding intimin and express a T3SS and effector proteins (encoded on the LEE pathogenicity island), but do not produce Stx. They are subdivided into strains harbouring the EPEC Adherence Factor plasmid (pEAF), which encodes the bundle forming pilus (BFP) required for localised adherence to epithelial cells) (typical EPEC, tEPEC) and strains lacking EAF plasmids (atypical EPEC, aEPEC). There is no clear evidence that all *eae*-positive *E. coli* are diarrhoeagenic. Current prevalence estimates amongst children with EPEC diarrhoea range from 6 to 54%. Recently, the Global Enteric Multicenter Study (GEMS), designed to detect the cause of paediatric diarrhoeal disease in sub-Saharan Africa and south Asia, found that infection with EPEC is associated with increased risk of fatality in infants aged 0–11 months (Kotloff et al., 2013).

Enteroinvasive *E. coli* (EIEC)

EIEC and *Shigella* spp. are so closely related that they should be classified as one distinctive species within the genus *Escherichia* (Brenner et al., 1972; Brenner et al., 1982). They are traditionally distinguishable by minor differences in biochemical characteristics. In a new multiplex PCR assay, an IpaH3 marker specific for *Shigella* spp. and one of five phylogenomic clade-specific markers have been combined for the rapid identification of *Shigella* spp. This assay appears to be able to distinguish between *Shigella* spp. and EIEC (Sahl et al., 2015). EIEC-*Shigella* invade gut epithelial cells by the trigger mechanism (Valencia-Gallardo et al., 2015), in a process mediated by invasion plasmid antigens (Ipa) encoded in the *ipa* operon that is in turn carried on a 220 kilobase (kb) virulence plasmid; the plasmid also encodes a type III secretion system (T3SS), that is responsible for injection of bacterial virulence factors into eukaryotic cells, where they subvert cell signalling to promote a favourable environment for bacterial growth and survival (Valencia-Gallardo et al., 2015). Illness is characterised by colitis and the appearance of blood and/or mucus in the faeces (van den Beld and Reubsaet, 2012).

Enterotoxigenic *E. coli* (ETEC)

ETEC is the most common enteropathogen found in children less than 5 years of age in low-income countries. It accounts for approximately 20% of diarrheagenic cases (Qadri et al., 2005), and has been estimated to be responsible for 6.0% of diarrhoeal disease-related deaths worldwide in children of less than 5 years of age between 1990 and 2011 (Lanata et al., 2013). ETEC is the most common cause of travellers' diarrhoea (*ca*. 10 million cases) accounting for 10–60% of infections depending on the region visited (Gascon et al., 1998). ETEC strains colonise the small intestine using one or more colonisation factor antigens (CFA). Diarrhoea is produced by heat-stable (ST) and/or heat-labile (LT) enterotoxins. It is estimated that ETEC causes several hundred million cases and several tens of thousands of deaths each year. ETEC causes disease in animals including cattle and neonatal and post-weaning pigs. Species specificity is mediated through acquisition of colonisation factors (CF) rather than appearance of animal-specific clones.

Diffusely Adherent *E. coli* (DAEC)

DAEC is a diverse group and its pathogenic potential remains uncertain. The pathotype is identified by the diffuse pattern of adherence to HEp-2 cells. Some reports suggested that DAEC might be an important contributor to paediatric diarrhoea or disease in children aged 18 months to 5 years (Gunzburg et al., 1993), although as at least one of the probes used for detection was of low specificity (Snelling et al., 2009), further epidemiological studies are required to establish the pathogenic potential of DAEC strains.

Adherent Invasive *E. coli* (AIEC)

AIEC express type I pili and Long Polar Fimbriae (LPF), which could mediate attachment of AIEC to the Peyer's patch M cells and tissue invasion. Such strains have been found associated with CD lesions in ileal and neo-terminal ileal and colonic specimens (van den Beld and Reubsaet, 2012). AIEC survive and replicate extensively without inducing host cell death and induce the release of high amounts of TNFα (Darfeuille-Michaud, 2002). As is the case for DAEC, it is still debatable whether there is a causative or symptomatic relationship between AIEC and CD. The cause and effect relationship between the presence of AIEC and CD has not yet been established, although an increased immune response to *E. coli* in CD patients suggests an involvement of *E. coli* in the pathology of CD, perhaps as a secondary coloniser of damaged areas of intestine.

Shiga toxin-producing E. coli (STEC)

STEC (also known as VTEC) strains are divided into those that encode intimin (*eae*-positive) and the LEE-encoded T3SS apparatus and effectors and those that are *eae*/LEE negative. STEC infections are prevalent in high-income countries and the most common serotype is O157:H7. Disease is mediated by colonisation factors and elaboration of Stx (mainly Stx1 and/or Stx2), which can cause bloody diarrhoea or haemolytic uraemic syndrome (HUS). Estimates made in 2011 in the United States suggest that 2,318 hospitalisations per annum are caused by STEC O157 (CDC, 2011). The most up to date evaluation of STEC infection was recently published by the EFSA BIOHAZ Panel (2013) as a Scientific Opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment.

Enteroaggregative *E. coli* (EAEC)

EAEC is the second most common cause of travellers' diarrhoea after ETEC (Shah et al., 2009) and its occurrence as endemic and epidemic disease is becoming well recognised. It causes persistent diarrhoea in children in low-income countries (Dutta et al., 1999; Sarantuya et al., 2004; Hebbelstrup Jensen et al., 2014; Benevides-Matos et al., 2015) and has been implicated as an important enteric pathogen affecting AIDS patients (Wanke et al., 1998). The 2011 German *E. coli* foodborne outbreak was caused by an EAEC strain (O104:H4) encoding Stx. EAEC are characterised by their ability to adhere to tissue culture cells in a distinctive stacked-brick pattern which is mediated by aggregative adherence fimbriae (AAF), of which there are several known isoforms (I, II, III, IV and V). Expression of AAF is mediated by the plasmid-encoded transcriptional activator AggR (Nataro et al., 1994; Dudley et al., 2006b). Cell attachment is also mediated by toxigenic invasion locus A (Tia). EAEC strains often produce an enteroaggregative heat-stable toxin (EAST1) encoded by the plasmid-borne *astA* genes as well as *Shigella* enterotoxin (ShET1) and Hemolysin E (HlyE) (Estrada-Garcia and Navarro-Garcia, 2012). In addition, EAEC strains encode a number of serine protease autotransporters of *Enterobacteriaceae* (SPATE) that are implicated in immune evasion, mucosal damage, secretogenicity, and colonization (Dutta et al., 2002). SPATEs include Plasmid-encoded toxin (Pet), Protein involved in intestinal colonization (Pic), Secreted autotransporter toxin (Sat), *Shigella* IgA-like protease homology (SigA) and *E. coli* secreted protein (EspP) (Boisen et al., 2009). EAEC strain O42 encodes a potentially functional but as yet uncharacterized T3SS, named ETT2 (Ren et al., 2004; Sheikh et al., 2006). In addition, EAEC strains encode two type VI secretion systems (T6SS), named $EAEC_{Sci\text{-}I}$ and $EAEC_{Sci\text{-}II}$ (Chaudhuri et al., 2010). T6SS are mainly used for interspecies bacterial completion (Russell et al., 2014). $EAEC_{Sci\text{-}II}$ is regulated by AggR and responsible for the secretion of a protein known as AaiC. The roles of the T3SS and T6SSs in the pathogenesis of EAEC are still not known. Santiago et al. (2014) described a novel EAEC regulator called Aar (AggR-activated regulator), which is a member of a previously unrecognized large class of regulators in pathogenic Gram-negative bacteria. The identified *aar* gene is activated by AggR, although when *aar* is deleted, *aggR* and the *aggR* regulon remain persistently activated. Thus, Aar can act directly or indirectly as a virulence suppressor as it

down-regulates the expression of the positive regulator AggR. Factors pertaining to the EAEC pathotype are listed in Table 3.

Extraintestinal pathogenic *E. coli* (ExPEC)

Extraintestinal pathogenic *E. coli* (ExPEC) encode virulence factors that allow it to invade, colonize, and induce disease outside of the gastrointestinal (GI) tract (Kohler and Dobrindt, 2011). Such strains include those causing urinary tract infections (UTIs) or or meningitis and sometimes are isolated from sepsis. Intestinal pathogenic *E. coli* are often epidemiologically and phylogenetically distinct from ExPEC and other non-pathogenic commensal strains, even though sometimes intestinal pathogenic *E. coli* may share with ExPEC the ability to cause UTIs, as in the case of the EAEC strains isolated from UTIs and bacteremia (Herzog et al., 2014).

Pathogenic *E. coli* with virulence characteristics from multiple pathotypes

The diarrhoeagenic pathotypes described above are, for the most part, defined on the basis of the presence of specific virulence features. *E. coli* strains possessing virulence genes typical of more than one pathotype may be isolated from cases of disease, thereby presenting difficulties in determining the aetiology of the disease and the sources of infection. Although not quantified, such pathotypes do seem to be increasingly common. The Stx-producing EAEC O104:H4, as well as other Stx-EAEC outbreak serotypes such as O111:H2 (Morabito et al., 1998), or HUS-associated serotypes O86:H- (Iyoda et al., 2000) or O111:H21 (Dallman et al., 2012), are examples of such disease-causing organisms. Other combinations have been detected, such as those present in isolates possessing EAEC-associated genes together with ExPEC-associated traits, as described in the *E. coli* serotypeO78:H10 responsible for causing an outbreak of UTI in Denmark (Olesen et al., 2012), and in non-H30 ST131 *E. coli* of serotype O25:H4 isolated from both UTIs and from patients with diarrhoea (Olesen et al., 2014). Finally, an ETEC strain from a case of diarrhoea was shown to possess the *stx2d*-encoding genes and to produce the toxin (Tozzoli et al., 2014).

Table 2: Genes and toxins often found in the EAEC pathotype

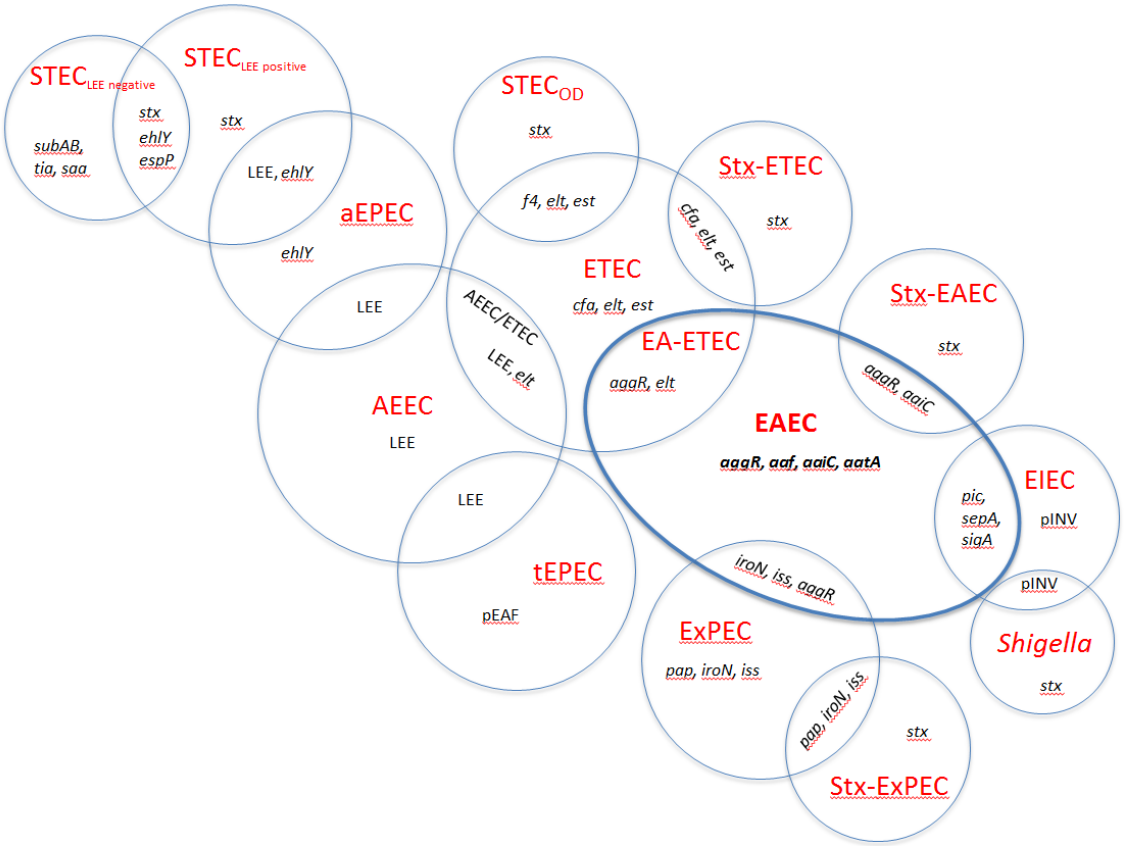
Common EAEC Factor	Description	Location	Presence in other pathotypes
Regulator genes			
aggR	Master regulator of a package of EAEC plasmid virulence genes, including aggregative adherence factors, fimbriae AAF/I-AAF/V, and a large cluster of genes inserted on a pathogenicity island at the PheU locus.	pAA plasmid	
aaR	AggR-activated regulator. Member of the family of AraC-Negative Regulators.	pAA plasmid	ETEC
AggR regulated genes			
aatA-P	Encodes ABC protein responsible for transporting the dispersin protein out of the outer membrane of EAEC.	pAA plasmid	ETEC
aap	Encodes a 10 kDa secreted protein named dispersin, and is responsible for 'dispersing' EAEC across the intestinal mucosa.	pAA plasmid	ND
aggA	Encodes AAF/I mediates adherence to colonic mucosa and haemagglutination of erythrocytes.	pAA plasmid	
aafA	Encodes AAF/II, mediates adherence to colonic mucosa and haemagglutination of erythrocytes.	pAA plasmid	
agg3A	Encodes AAF/III haemagglutination of erythrocytes.	pAA plasmid	
agg4A	Encodes AAF/IV mediates adherence to colonic mucosa and haemagglutination of erythrocytes.	pAA plasmid	
agg5A	Encodes AAF/V mediates adherence to colonic mucosa and haemagglutination of erythrocytes.	pAA plasmid	
aaiC	Encodes AaiC, secreted protein. Type 6 secretion system. Mode of action unknown.	Chromosome	
ORF3/4	Co-regulated two-gene cluster with homology to isoprenoid synthesis genes.	pAA plasmid	ND
capU	Encodes Hexosyltransferase homologue.	Chromosome	ND
Serine Protease Autotransporters (SPATEs)			
Pet	A 108 kDa autotransporter protein that functions as a heat-labile enterotoxin and cytotoxin.	pAA plasmid	
SigA	IgA protease-like homologue, enterotoxin and cytotoxin.	Chromosome	EIEC
Pic	Mucinase, immunomodulation, colonization, lectin-like haemagglutinin.	Chromosome	EIEC
SepA	*Shigella* extracellular protein. Enterotoxin.	pAA plasmid	EIEC, EPEC
Sat	Secreted autotransporter toxin. Enterotoxin and cytotoxin, impairment of tight junctions, autophagy.	pAA plasmid	UPEC, DAEC
Enterotoxins		Chromosome	
EAST1	*astA* encodes the enteroaggregative heat-stable toxin (EAST1), which has physical and mechanistic similarities to *E. coli* STa enterotoxin.	pAA plasmid	ETEC, EPEC, STEC, UPEC
Other toxins			
eilA	*Salmonella* HilA homologue, activates the bacterial surface protein Air.	Chromosome	ND
air	Possible aggregation and adherence.	Chromosome	ND

AAF: aggregative adherence fimbriae; DAEC: diffusely adherent *E. coli*; EAEC: enteroaggregative *E. coli*; EIEC: enteroinvasive *E. coli*; EPEC: enteropathogenic *E. coli*; ETEC: enterotoxigenic *E. coli*; ND: Not determined; STEC: Shiga toxin-producing *E. coli*; UPEC: uropathogenic *E. coli*.

3.1.3. Interrelationships between *E. coli* pathotypes

Pathogenic *E. coli* have been historically identified on the basis of their ability to produce toxins or by virtue of their specific abilities in the colonization of the host (see Section 3.1.2).

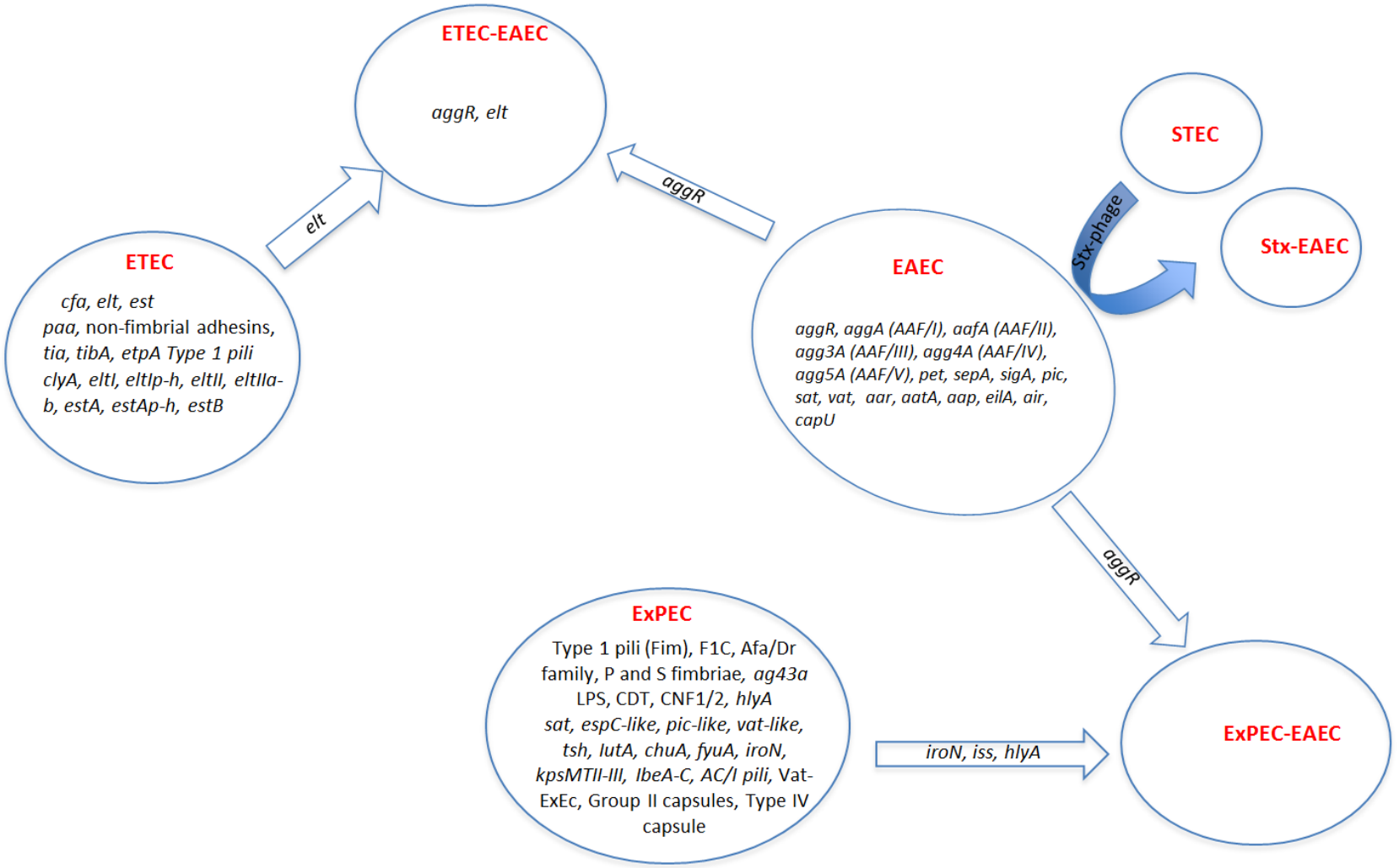
As knowledge has advanced, supported by the development of investigative technologies based on molecular biology and genomics (Franz et al., 2014), new pathotypes have been defined and it is now clear that the spectrum of pathogenic *E. coli* types is continuous rather than a rigid list of separated groups. This dynamic continuum can be depicted as a bubble chart which allows for new pathotypes to be included as they emerge (Figure 1). Most of the *E. coli* virulence factors are encoded by genes carried on mobile genetic elements (e.g. plasmids, phages and pathogenicity islands). Indeed, horizontal gene transfer of such elements powers *E. coli* genomic plasticity and is the driver for the continuous emergence of new pathotypes.



Size, shape and distance between the bubbles do not have any phylogenetic significance. AEEC indicates *the E. coli* strains causing the attaching and effacing lesion and designate a super-group of LEE-positive *E. coli*. $STEC_{OD}$ indicates those strains causing the oedema disease in pigs.

Figure 1: Bubble chart illustrating the *E. coli* pathotype continuum

The appearance of the Stx-producing EAEC O104:H4 boosted studies on the identification of *E. coli* strains with cross-pathotype arrays of virulence genes. Reports of *E. coli* strains displaying virulence features that did not fit the pathotypes scheme date back to the late 1990s with the first description of an Stx-producing EAEC during the investigations of an HUS outbreak in France (Morabito et al., 1998). Other reports included strains hosting virulence genes of ETEC and EAEC (Fujioka et al., 2013) or STEC strains producing stx2e and the heat stable and heat labile toxins typically produced by ETEC (Mainil and Fairbrother, 2014) or ETEC strains harbouring an Stx2 phage (Tozzoli et al., 2014). EAEC showing different arrangements of virulence genes include those with determinants associated with ExPEC, EIEC, ETEC, and STEC (Figure 2). This list is not complete and will probably grow as the capacity to study the *E. coli* genome increases, for example by the use of Whole Genome Sequencing (WGS) (Appendix F).



The white arrows indicate the transfer of genetic determinants between EAEC and other pathogenic *E. coli*. The solid arrow indicates the acquisition of a Stx-converting bacteriophage. The *E. coli* pathotype represented by each bubble is indicated in red, while the genes identifying the single pathogenic groups or exchanged between groups of *E. coli* are shown in black.

Figure 2: Schematic illustration of the possible interrelationships between EAEC and other pathogenic *E. coli*

3.1.4. Molecular structure of EAEC

Genomics has made an important contribution to our understanding of the evolution and pathogenic potential of EAEC. EAEC are phylogenetically diverse and have a heterogeneous virulence gene profile. EAEC lineages have evolved independently many times *via* multiple genetic events. The mosaic genomic structure facilitates horizontal gene transfer, which is the driving force for acquisition of novel genome features and potentially novel pathogenic mechanisms. Subsequent recombination and multiplication results in the integration of such elements into the genome. The EAEC pan-genome is considered open and is still evolving by gene acquisition and diversification. This has public health implications resulting from the diversity and pathogenesis of EAEC and their ability to colonise and cause disease in the human host (Appendix F).

3.1.5. Conclusions

Gastroenteric *E. coli* strains are conventionally divided into six pathotypes based on their pathogenicity profiles. These are: enteroaggregative *E. coli* (EAEC), EPEC, STEC, EIEC (including *Shigella* spp.), ETEC and DAEC.

As some pathotypes can belong to more than one serotype, serotyping may not provide definitive identification of pathotypes.

An individual *E. coli* strain possessing virulence genes typical of more than one pathotype may be isolated from cases of disease, thereby presenting difficulties in epidemiological investigation.

EAEC are characterised by their ability to adhere to tissue culture cells in a distinct stacked-brick pattern which is mediated by aggregative adherence fimbriae (AAF), of which there are several known isoforms (I, II, III, IV and V).

Pathotype-determining factors for EAEC include *aggR, aaiC, aggA, aafA, agg3A, agg4A, agg5A,* AAF/I-V.

Expression of AAF is mediated by the plasmid-encoded transcriptional activator AggR; cell attachment is also mediated by the Toxigenic invasion locus A (Tia).

EAEC often produce an enteroaggregative heat-stable toxin (EAST1) encoded by the plasmid-borne *astA* genes as well as *Shigella* enterotoxin (ShET1) and Haemolysin E (HlyE).

EAEC also possess genes that encode a number of serine protease autotransporters of *Enterobacteriaceae* (SPATE) that are implicated in immune evasion, mucosal damage, secretogenicity, and colonization.

The acquisition of virulence traits present in other pathotypes by EAEC can result in the formation of new, highly virulent organisms.

EAEC lineages have evolved independently many times *via* multiple genetic events. The EAEC pan-genome is considered open and is still evolving by gene acquisition and diversification.

4. Public health significance of EAEC related to the handling, preparation and consumption of food (ToR 2)

4.1. Occurrence of EAEC in animals

Reports of animals being a reservoir of EAEC are often based on the presence of genes such as *astA*, which are not specific for EAEC (Veilleux and Dubreuil, 2006), in specimens from both healthy and sick animals. Most reports originate from parts of the world where pollution by human faecal waste is common, such as low-income countries in Africa and South America. There is no evidence in the EU for animals being a relevant reservoir of EAEC.

Virulence genes specific for five major pathotypes of *E. coli* in primary cultures from faeces of animals slaughtered for human consumption in Burkina Faso were investigated. Faeces were collected from cattle (n = 304), chickens (n = 350), and pigs (n = 50) after slaughter. Virulence genes specific for different pathotypes were detected in the following percentages of the cattle, chicken, and pig faeces

samples: STEC in 37%, 6%, and 30%; EPEC in 8%, 37%, and 32%; ETEC in 4%, 5%, and 18%; and EAEC in 7%, 6%, and 3.2% (Kagambega et al., 2012c).

A total of 263 *E. coli* isolates were isolated from South African pigs aged between 9 and 136 days; PCR was used in the analysis. Virulence genes were detected in 40.3% of the isolates, of which 18.6, 0.4 and 17.5% were classified as ETEC, STEC and EAEC, respectively. Individual genes were found in the following proportions: STb (19.01%), LT (0.4%), STa (3.4%), St2xe (1.1%) and EAST1 (20.2%) toxins, but more specific assays for EAEC-related genes would be needed to confirm the true prevalence (Mohlatlole et al., 2013). Similar observations were made regarding the involvement of putative EAEC in enteritis of sucking piglets in Poland (Weiner et al., 2004).

In Zambia, the EAST1 gene was detected more frequently in *Kafue lechwe* antelopes (83.3%) than in pastoral cattle (33.3%) specimens ($p < 0.01$). In contrast, the Stx2-positive samples in *Kafue lechwe* (20.0%) had a significantly lower percentage than that in cattle (55.1%, $p < 0.01$). It was postulated that these animals could be reservoirs of EAEC, but more specific tests would be required to confirm this (Kuroda et al., 2013).

Another study in Brazil, a country where EAEC is prevalent in humans of lower socio-economic groups, especially children, compared the phenotypic (serotype) and genetic (ribotype and virulotype) similarity of human and animal EAEC strains and found little evidence of overlap (Uber et al., 2006).

Two distinct diarrhoeagenic *E. coli* pathotypes, EAEC and STEC, were observed in association with *E. coli* O113 isolates from human and non-human sources in Brazil, respectively. The *E. coli* O113 isolates from cases of human diarrhoea belonged to a diversity of serotypes, nine (53%) of which harboured virulence traits of EAEC. It was not possible to conclude from this study that there were non-human sources for the EAEC strains (dos Santos et al., 2007).

The virulence attributes of a collection of 56 *E. coli* isolates from sick horses in Brazil (secretions of uterine cervices, GI and lung fragments of necropsy, diarrheic faeces, and tracheal washings) was examined by determining their adherence pattern to HeLa cells and searching for the presence of virulence genes of the various *E. coli* pathotypes. Two non-adherent strains presented *astA*, which encodes the EAEC heat-stable toxin. Twenty-seven strains (48.2%) adhered to HeLa cells, 21 (77.8%) of which presented the AA pattern that characterize the EAEC pathotype. Nine of the strains presenting AA were isolated from secretions of uterine cervix, including one carrying virulence genes of the EAEC pathotype (*aggR*, *aap*, *irp2*, and *pic*) (Alvim Liberatore et al., 2007). Putative EAEC have been isolated from diarrhoeic dogs in Germany (Breitwieser, 1999). No EAEC strains were found in a study of 24 sick Brazilian parrots (Knoebl et al., 2011), but one EAEC strain was identified from bacterial cultures of cloacal swabs from 86 captive kept psittacine birds in Brazil (Marietto-Goncalves et al., 2011). EAEC have been isolated from dogs and cats and at low levels from poultry manure in Brazil (Puno-Sarmiento et al., 2013; Puno-Sarmiento et al., 2014). *E. coli* carrying the *astA* gene were isolated from a dead duck in India (Subodh et al., 2013).

PCR assays and culture were used to investigate 728 faecal samples from 404 calves (286 diarrhoeic, 118 healthy) and 324 lambs (230 diarrhoeic, 94 healthy) in Kashmir, India; no EAEC was identified (Wani et al., 2013). A survey of 1,227 caecal *E. coli* isolates from healthy cattle, sheep and pigs at slaughter in UK was was undertaken in 2003 using a pAA probe target. No EAEC were found (Cassar et al., 2004).

A study of isolates from piglet diarrhoea cases in eastern Europe suggested the possible involvement of EAEC, as 34.6% of the isolates possessed the *astA* gene, encoding EAST1 toxin considered at that time to be a genetic marker for EAEC isolates. This marker was almost exclusively present only among ETEC strains, and the study did not conclusively demonstrate the presence of EAEC (Dacko et al., 2004).

In France, 10,618 *E. coli* isolates from abattoir waste discharge were screened by PCR for the presence of EAEC-associated genetic markers (*aggR*, *aap* and *aatA*). None of these markers was detected amongst the *E. coli* isolated from slaughterhouse samples. A novel EAEC O126:H8 was detected in river water sampled upstream from slaughterhouse effluent discharge, indicating a different source. These results provide further evidence that animals are unlikely to be major reservoirs of EAEC in the EU (Bibbal et al., 2014).

To clarify whether the Stx-producing EAEC O104:H4 outbreak strain and/or EAEC may be able to colonize ruminants, 2,000 colonies from faecal samples of 100 cattle from 34 different farms, all located in the HUS outbreak region of Northern Germany, were screened for genes associated with the O104:H4 HUS outbreak strain (*terD*, *rfb*(O104), *fliC*(H4)), STEC (*stx1*, *stx2*, *escV*), EAEC (*pAA*, *aggR*, *astA*), and ESBL-production (*bla*(CTX-M), *bla*(TEM), *bla*(SHV)). The faecal samples contained neither the HUS outbreak strain nor any EAEC. None of the selected strains showed an aggregative adhesion pattern on HeLa cells (Wieler et al., 2011). In a similar study undertaken in France after the 2011 outbreak, 1,468 cattle were analysed for faecal carriage of the Stx-producing *E. coli* O104:H4 outbreak strain by PCR assays targeting *stx2*, *wzxO104*, *fliCH4* and *aggR* genetic markers. None of the faecal samples contained the four markers simultaneously, indicating that cattle in France were not likely to be a reservoir of O104:H4, but results of the test for *aggR* were not reported (Auvray et al., 2012). In a recent study of 100 clinical cases in cattle in Japan, no EAEC isolates, as assessed by the presence of *aggR,* were detected (Akiyama et al., 2015).

A mouse *in vivo* challenge model is often used to mimic human infection as characteristics of infection are considered to be similar (Roche et al., 2010; Maura et al., 2012; Saha et al., 2013). In another study, type strains of EAEC (17-2, serotype O3:H2; JM 221, serotype O92:H33), isolated from human infections were used to orally infect adult rabbits. This led to intestinal colonisation and some enteritis (Kang et al., 2001). Wild rabbits have been shown to carry STEC O157 strains under natural conditions when exposed to infected cattle (Pritchard et al., 2009).

Use of the German outbreak strain of Stx-producing EAEC O104:H4 in an infant rabbit-based model of intestinal colonization and diarrhoea suggested that the pAA virulence plasmid is not essential for intestinal colonization and development of intestinal pathology. Instead, chromosome-encoded auto-transporters were critical for robust colonization and diarrhoeal disease in the rabbit model (Munera et al., 2014). In a further study for EAEC pathogenesis in two animal models using *E. coli* O104:H4 with and without *stx* genes, it was shown that Stx2 is responsible for most of the virulence observed in C227-11-infected mice and rabbits (Zangari et al., 2013).

There are concerns about the validity of findings from animal models in the case of EAEC and the duration of colonisation can be short (Sainz et al., 2002), but such models do confirm the potential for colonisation of various animal species if a substantial challenge dose is given (Philipson et al., 2013).

4.2. Occurrence of EAEC in food (food-borne transmission of EAEC)

There is evidence in the literature of foodborne transmission of EAEC, mostly through documented outbreaks and case-control studies. In India, a village-wide outbreak in 1996 was epidemiologically-associated with the consumption of water from open wells (Pai et al., 1997). In Japan a major outbreak in 1993 involving up to 2,500 cases mainly in school children was associated with school lunches (Itoh et al., 1997). In this outbreak the causative strain was identified as *E. coli* O untypeable:H10. A further EAEC food-poisoning outbreak in Shizuoka, Japan, in 2005 was reported by Harada et al. (2007). In this outbreak samples were obtained from five patients and two food handlers, and the causative strain was O126:H27.

In the UK at least four EAEC outbreaks associated with restaurants, a charity Christmas dinner and a conference have been reported but no food sources were identified (Smith et al., 1997). A further case-control study in the UK found a strong association with travel abroad and for those who did not travel abroad, eating salad at a restaurant was associated with a four-fold increase in risk of EAEC infection (FSA, 2000). The 2011 German outbreak of EAEC O104:H4 (see Section 1.1.3 above) was epidemiologically-linked to contaminated fenugreek seeds (EFSA, 2011b). In June 2013, a food-borne outbreak was caused by an EAEC of unknown serotype (ONT:H-) and isolated from kippered trotters (a smoked pigs' feet delicacy) mixed with vegetables, 22 cases and four asymptomatic food handlers, which probably contaminated the food (Shin et al., 2015). This outbreak highlights the importance of safe food preparation.

EAEC virulence genes were only detected in combination with those of EPEC (on 11 carcasses) or STEC (on 2) in a survey of 100 poultry carcases purchased from three street markets selling poultry in Ouagadougou, Burkina Faso (Kagambega et al., 2012a). EAEC virulence markers (*plc* or *aggR*) were found in 5 (4%) of 120 samples of beef and edible intestines from such markets (Kagambega et al., 2012b). Conversely, no EAEC were found amongst 162 *E. coli* isolates from foods (raw meat, fish, and

processed foods) that were collected in Korea (Koo et al., 2012). The infection status of food handlers, including asymptomatic carriage of EAEC, and hygienic conditions applied during the handling and processing of foodstuffs in some countries seems to be an important factor in contamination of foods at retail, catering or household level (Gilligan, 1999; Oundo et al., 2008).

Outbreaks of *E. coli* with properties of EAEC, either alone or mixed with other pathogens, have been reported from a number of countries and have involved different food vehicles, including cheese made from unpasteurised milk and fresh produce (Hedberg et al., 1997; Smith et al., 1997; Harada et al., 2007; Scavia et al., 2008; PHE, 2013). For example, an outbreak of GI illness in April 1991 among patrons of a restaurant in the USA was caused by a strain of *E. coli* O39:NM, which contained the LEE and was also found to produce EAST1. The strain was isolated from affected individuals but not from foods (Hedberg et al., 1997). In two further foodborne outbreaks of gastroenteritis that occurred 10 days apart among individuals who had meals at the restaurant of a farm holiday resort in Italy in 2007, an EAEC strain of serotype O92:H33 was isolated from six participants and one member of staff. A retrospective cohort study indicated a pecorino cheese made with unpasteurized sheep milk as a possible source of infection (Scavia et al., 2008), but since the outbreak EAEC strain was only isolated from food handlers, cross-contamination of the food product cannot be excluded, nor can contamination of food by asymptomatic excretors.

More recently, in a study of ESBL-producing *Enterobacteriaceae* in different types of fresh vegetables imported into Switzerland, an EAEC isolate belonging to the multilocus sequencing type (MLST) D:ST38 lineage was identified in okra originating in India (Zurfluh et al., 2015). The authors of the study commented that the detection of such a pathogen in vegetables destined for human consumption raises questions concerning food safety.

4.3. Outbreaks of gastrointestinal disease associated with multiple gastrointestinal pathogens including EAEC

An increasing number of diagnostic microbiology laboratories are implementing a multiplex gastrointestinal (GI) PCR approach for the detection of GI pathogens in clinical cases and foods. These assays provide a rapid, and cost-effective pan-pathogen approach for the detection of bacteria, viruses and parasites commonly associated with GI infection (Moran-Gilad et al., 2012). Micro-array based approaches have also been developed and an example of this technology is the FilmArray GI Panel, which is the most comprehensive, low cost, multiplex diagnostic test currently available (22 target GI pathogens) and the results are available in one hour with minimal hands-on processing time. Although such multiplex tests will increase both the detection rate and the number of co-infections that are identified, routine bacterial culture will still be needed for antimicrobial susceptibility testing and epidemiological investigations (Binnicker, 2015).

The pan-pathogen GI PCR approach has already provided evidence that episodes of diarrhoea can be caused by more than one pathogen (Wilson et al., 2001; Tam et al., 2012a; Kotloff et al., 2013; Spina et al., 2015). Proving specifically which pathogens are most likely to have contributed to clinical symptoms can be difficult. For example, in an outbreak of GI foodborne illness associated with a Street Spice festival in the UK in 2011 and involving over 400 persons, 29 cases of *Salmonella* infection were confirmed. As most cases had reported symptoms characteristic of EAEC infection such as abdominal cramps and persistent diarrhoea, further investigations using a PCR assay demonstrated that EAEC and *Shigella* spp. were present in many of patients and may have contributed to the outbreak (Dallman et al., 2014). Risk factors associated with illness included eating foods from one particular vendor and eating a food item containing uncooked curry leaves. Although the *E. coli* count in colony forming unit (cfu) per mL from the curry leaves associated with the outbreak was high (> 1,000 cfu/mL), the testing algorithm at that time did not include tests specific for EAEC and EAEC was not cultured from the food samples.

Two other multi-pathogen outbreaks involving EAEC include 39 cases identified following a school trip to Morocco in October 2013, and four cases of GI illness with an associated high fever identified among eight UK Ministry of Defence healthcare personnel who had recently returned from active duty at an Ebola treatment centre in Sierra Leone. In both outbreaks, symptoms of persistent diarrhoea and abdominal cramps raised the suspicion that some cases may have infection with EAEC. In the outbreak associated with travel to Morocco, initial laboratory reports identified two enteric pathogens, *Shigella sonnei* and *Campylobacter* spp., but symptoms of persistent diarrhoea and abdominal cramps

raised the suspicion that some cases may have infection with EAEC, which has been associated with travellers returning from North Africa (Perry et al., 2010). Because of this a multiplex PCR GI assay was performed, which confirmed the presence of EAEC. In the outbreak associated with Sierra Leone, PCR results from stool samples suggested that all eight cases were *Shigella*-positive, two were EPEC-positive and another two were EAEC-positive. Food contaminated by a food handler was identified as a potential source of the infection in both outbreaks.

4.4. Occurrence of EAEC in the environment

Multiplex PCR was used in a survey of natural water in Bangladesh. Genes from the ETEC, EPEC and STEC pathotypes were detected consistently, but genes from the EIEC and EAEC pathotypes were only found occasionally, and never in the rainy season or in winter (Akter et al., 2013). EAEC was found in 16% of 80 samples from domestic rainwater harvest tanks during a longitudinal study in a high-population-density, urban setting and in river water samples in South Africa (Obi et al., 2004; Dobrowsky et al., 2014). In a study of pre-treated water in a drinking water treatment plant in Taiwan, EAEC-associated genes were found in 3.6% of 55 water samples, alongside with high levels of other potentially pathogenic *E. coli* (Huang et al., 2011). In a sub-tropical area of Australia, *astA* (69%) and *aggR* (29%) genes, carried by EAEC, were frequently detected in *E. coli* isolates from urban floodwater (Sidhu et al., 2013).

Various EAEC infections have been traced back to countries where EAEC infections are endemic and treatment of human sewage is poor or non-existent. Such countries may represent a reservoir for the emergence of the Stx-producing *E. coli* pathotype since EAEC of human origin can extensively contaminate the environment where they can combine with free *stx*-carrying phages from ruminant faecal waste (Trivenee et al., 2012; Grande et al., 2014).

The occurrence of EAEC in flies is likely to reflect their recent exposure to environmental contamination and may result in onward transmission (Forster et al., 2009). The large numbers of *Chrysomya putoria* that can emerge from pit latrines, the presence of enteric pathogens on flies, and their strong attraction to raw meat and fish suggests these flies may be common vectors of diarrhoeal diseases in Africa (Lindsay et al., 2012).

4.5. Factors affecting the survival of EAEC

Various non-spore forming bacteria, including *E. coli*, can enter a dormant-like state - the viable but non-culturable (VBNC) state -, characterized by the presence of viable cells but the inability to grow on routine laboratory media (Li et al., 2014). Upon resuscitation, these VBNC cells may recover both culturability and pathogenicity (Pommepuy et al., 1996). The ability of the Stx-producing EAEC O104:H4 outbreak strain to enter the VBNC state may have complicated its detection in the suspected food sources. In a nutrient-poor micro-environment such as water, cells may become non-culturable within a few days, but may remain viable for several weeks, especially at low temperatures (Aurass et al., 2011). In contrast, in another study, EAEC strains were able to survive, and possible multiply at higher temperatures, for at least 60 days in bottled mineral water or spring water (Vasudevan et al., 2003) and more definitive studies are required in this area.

Survival of EAEC in the dry conditions of milk powder factories has also been reported, but the origin of the contamination was not attributed to the milk itself (Duffy et al., 2009). It is thought that prolonged survival of organisms on dry fenugreek seeds may have been involved in the Stx-producing EAEC O104:H4 outbreak (EFSA, 2011b; Soon et al., 2013).

When mung bean seeds were artificially inoculated with EAEC, the strain survived at least 90 days at 25°C, there was further growth during germination and sprouting of seeds, reaching counts of approximately 5 log cfu/g after 2 days at 20°C. When the sprouts were inoculated after 1 day of germination and stored at 20°C no growth was observed during further sprouting, suggesting a protective effect, but growth did still occur at 30°C, demonstrating the importance of temperature-controlled storage in hot climates (Gomez-Aldapa et al., 2013).

An experimental study of factors contributing to the ability of EAEC to attach to salad leaves showed that multiple adherence factors are involved in the interaction of EAEC with leaves, and that similar colonization factors are used to bind to mucosal and leaf surfaces (Berger et al., 2009).

4.6. Biofilm formation by EAEC

Bacterial biofilms are structured communities of bacterial cells enclosed in a self-produced polymer matrix (consisting of proteins, exopolysaccharide and nucleic acid) that is attached to biotic and abiotic surfaces. Biofilms protect and allow bacteria to survive and thrive in hostile environments as well as facilitate chronic/persistent infections. Bacteria within biofilms can withstand host immune responses, and are much less susceptible to antimicrobials and disinfectants when compared to their planktonic counterparts (Tremblay et al., 2014).

EAEC form thick biofilms on the intestinal mucosa and most EAEC strains form a biofilm on glass or plastic surfaces when grown in cell culture medium with high sugar and osmolarity. Biofilm-forming ability is associated with expression of AAF, of which there are five variants (AAF/I-AAF/V), although many EAEC strains that do not express AAF developed biofilms under these conditions. AAFs bind extracellular matrix proteins and show species-specificity in terms of erythrocyte agglutination, suggesting that this binding specificity could impact on the efficiency and selectivity of biofilm formation.

Transposon mutagenesis confirmed the involvement of genes known to be required for AAF/II expression, as well as the *E. coli* chromosomal *fis* gene, a DNA-binding protein that is involved in growth phase-dependent regulation. Using reverse transcription-polymerase chain reaction (RT-PCR), the effect of *fis* was found to be at the level of transcription of the AAF/II activator *aggR*. Biofilm formation also required the product of the *yafK* gene that encodes a protein required for transcription of AAF/II-encoding genes. EAEC strains appears to form biofilm, which may be mediated by AAF, to varying degrees and this may influence their interactions with the intestinal mucosa (Sheikh et al., 2001).

E. coli O104:H4 produces a stable biofilm *in vitro*, and *in vivo* virulence gene expression is greater when the organism overexpresses genes required for aggregation and exopolysaccharide production, a characteristic of bacterial cells persisting within an established biofilm (Al Safadi et al., 2012).

Using X-ray crystallography and Nuclear Magnetic Resonance (NMR) structures, Berry et al. (2014) identified variability in the structure of AAF variant I from the O104 strain, and AAF variant II from archetype strain O42. The studies showed that AAF-fibronectin attachment is based primarily on electrostatic interactions, a mechanism not reported previously for bacterial adhesion to biotic surfaces (Berry et al., 2014).

The incompatibility group (Inc) I1 plasmid of EAEC C1096 encodes a type IV pilus that contributes to plasmid conjugation, epithelial cell adherence, and adherence to abiotic surfaces, including *via* biofilm formation (Dudley et al., 2006a). Other studies suggest that the Shf protein-encoding gene (*shf)* is required for strong biofilm formation of EAEC serotype O42, and that transcription of the *shf* gene is dependent on AggR (Fujiyama et al., 2008). There is considerable heterogeneity in putative virulence genes of EAEC isolates from diarrhoeic children in India and biofilm formation appears to be associated with multiple genes (Wani et al., 2012).

When subjected to low iron conditions, an EAEC O42 strain showed a decrease in biofilm formation. Conversely, an increase in biofilm formation was observed for clinical EAEC strains cultured in restricted iron conditions, but the reduction of iron concentration inhibited the aggregative adherence to HEp-2 cells of all EAEC strains tested. Low iron availability may therefore modulate biofilm formation and adhesive properties of EAEC, as a result of redox stress (Alves et al., 2010). Biofilm formation has been proposed as a cheap and rapid assay for EAEC (Wakimoto et al., 2004; Bangar and Mamatha, 2008).

AAF-mediated adhesion and biofilm formation is likely to be involved in both clinical manifestations of infection and attachment to foodstuffs, such as lettuce after irrigation or washing using water that has become contaminated with human faecal waste (Mendez-Arancibia et al., 2008; Berger et al., 2009; Castro-Rosas et al., 2012; Bolick et al., 2013; Berry et al., 2014). Uropathogenic strains in particular may make use of biofilm formation to persist on epithelial surfaces and canulae (Boll et al., 2013; Hebbelstrup Jensen et al., 2014), but a high proportion of EAEC strains associated with travellers' diarrhoea produce biofilms, as well as being highly antimicrobial-resistant (Mohamed et al., 2007; Mendez Arancibia et al., 2009). A synergistic effect, leading to substantially enhanced adhesion, has

been observed in mixed biofilms involving EAEC and *Citrobacter freundii*. This appeared to be mediated by pili that can be inhibited by the presence of zinc (Pereira et al., 2010).

Depending on the concentration, *Lactobacillus casei* inhibited biofilm formation of the majority (> 80%) of EAEC strains from human chronic inflammatory bowel disease, although a few strains (approximately 18%) formed biofilm regardless of the presence and concentration of the probiotic *L. casei* strain (Andrzejewska and Sobieszczanska, 2013). Defatted milk, whey proteins, immunoglobulin and non-immunoglobulin fractions, in concentrations lower than usually found in whole milk, also inhibit EAEC adhesion to HeLa cells (Araujo and Giugliano, 1999).

4.7. Clinical symptoms and severity of illness

While not all strains are diarrhoeagenic, EAEC have been commonly associated with acute diarrhoeal illness among children in both developing (Pai et al., 1997; Okeke et al., 2000; Bouzari et al., 2001; Sarantuya et al., 2004; Araujo et al., 2007) and developed/industrialised regions (Smith et al., 1997; Presterl et al., 1999; Knutton et al., 2001; Nataro et al., 2006), with travellers' diarrhoea (Adachi et al., 2001) and with diarrhoeal infections in persons with HIV (Nataro, 2005; Cennimo et al., 2007; Croxen et al., 2013; Hebbelstrup Jensen et al., 2014). Cases of both acute and persistent diarrhoea have been described. The latter usually occur in children less than a year old (Greenberg et al., 2002). Indeed, a predominant feature of EAEC infection in low-income countries is the propensity to cause persistent diarrhoea for more than 2 weeks, making these bacteria a significant cause of mortality (Huang et al., 2007).

The incubation period of diarrhoeagenic EAEC is typically between 8 and 18 hours (Nataro et al., 1998; Harrington et al., 2006), although an incubation period of between 40 and 50 hours for the onset of GI illness has been reported in at least one major outbreak (Itoh et al., 1997). Infection with EAEC usually presents clinically as watery diarrhoea, often with mucus, nausea and vomiting (Huang and Dupont, 2004; Huang et al., 2007; Scavia et al., 2008), but rarely accompanied by blood or mucus (Cennimo et al., 2007). While the majority of patients do not show signs of fever (Nataro, 2005), a low grade fever has been reported in a small percentage of cases (Huang et al., 2006). Other, less common, symptoms include anorexia, borborygmi, and tenesmus. In a limited number of cases, patients present with mucoid stools with or without blood (Kahali et al., 2004; Nataro et al., 2006). Additionally, the odds of developing post-infectious irritable bowel syndrome (IBS) are dramatically increased after acute infectious gastroenteritis (Okhuysen et al., 2004; Thabane et al., 2007).

Several studies have supported the association of EAEC with persistent diarrhoea in low-income countries (Bhan et al., 1989; Fang et al., 1995). In Brazil, EAEC infection is the most common cause of diarrhoea in small children, particularly those less than 2 years of age (Araujo et al., 2007). The most significant public health concern stemming from EAEC infections in children in low-income countries is malnourishment, as persistent EAEC infections lead to chronic inflammation, which damages the intestinal epithelium and reduces its ability to absorb nutrients (Vial et al., 1988).

Other studies suggest EAEC are a major cause of diarrhoeal disease in high-income countries (Presterl et al., 1999; Knutton et al., 2001) and several outbreaks both in children and in adults have been described in different regions of the world (Presterl et al., 1999; Adachi et al., 2001; Ruttler et al., 2002). It has been estimated that between 2% and 68% of patients with diarrhoea are infected with EAEC (Nataro et al., 1998; Presterl et al., 2003; Kahali et al., 2004). In the UK IID study in 1993–1996, EAEC were the most commonly isolated enterovirulent *E. coli* in patients with symptoms of gastroenteritis presenting to a doctor (5.1%) (FSA, 2000; Evans et al., 2002). In the second IID study in 2008–09, EAEC were isolated from more than 1.9% of cases in the population and 1.4% of cases presenting to a doctor (Tam et al., 2012a; Tam et al., 2012b). Based on more recent studies of EAEC isolates from the two IID studies, Chattaway et al. (2013) have concluded that the current definition of EAEC by plasmid gene detection includes true pathogens as well as non-pathogenic variants.

Stx-producing EAEC have also been associated with HUS and haemorrhagic colitis (HC) in addition to the EAEC diarrhoeal symptoms described above. Such symptoms were clearly evidenced in an outbreak of E*. coli* O111:H2 in France in 1992 (Boudailliez et al., 1997; Morabito et al., 1998).

Studies by Nataro (2005) to investigate EAEC pathogenicity have suggested that strains carrying the genes encoding AAF/II fimbriae and EAST1 are more likely to be associated with diarrhoeal disease.

In outbreaks of infection associated with HUS and HC such as those described above, the infecting strains have additional virulence factors such as *stx* genes (Morabito et al., 1998; Bielaszewska et al., 2011).

During infection, EAEC bind mainly to the mucosa in the colon and the terminal ileum but to a lesser extent in the small intestine, stimulating the epithelial cells to produce a thick mucus layer. Formation of this thick mucus gel on the intestinal mucosa, often with mucosal damage caused by toxins, are key pathogenic features of EAEC histopathology. A study by Roche et al. (2010) reported growth retardation in mice due to EAEC infection. The extent of retardation was dependent on the dose of bacteria used and malnourished mice had increased shedding of EAEC in their stools.

In immunocompetent individuals EAEC may be carried asymptomatically (Nataro et al., 2006; Tam et al., 2012a; Tam et al., 2012b; Chattaway et al., 2013) and in immunocompromised individuals (e.g. HIV) infection may be associated with more severe disease (Hebbelstrup Jensen et al., 2014).

Genetic factors within the host are important in determining susceptibility to EAEC infection and the clinical manifestations of EAEC infection vary from individual to individual, depending upon the genetic composition of the host (Jiang et al., 2003). Mutations in the interleukin (IL)-8 gene promotor and the lactoferrin gene are known to be associated with greater susceptibility to EAEC infection (Jiang et al., 2003; Mohamed et al., 2006). Specifically, the presence of an AA genotype at the 251 position in the IL-8 promoter region homozygous for a single nucleotid polymorphism (SNP) produces higher levels of faecal IL-8. These patients develop symptomatic EAEC diarrhoea more frequently than those heterozygous for the gene. Furthermore, SNPs in the regulatory and codon regions of a number of cytokine genes such as tumour necrosis factors (TNF), IL-1, IL-1ra, IL-4, and IL-6 have been shown to have an impact on the type of immune response and the severity of disease associated with EAEC infection (Rosenwasser and Borish, 1998).

4.8. Extra-intestinal EAEC infections

Several studies have reported EAEC-defining genes in uropathogenic *E. coli* isolates. A study among children in Nigeria linked EAEC to the new uropathogenic clonal group A (Wallace-Gadsden et al., 2007), and a study in Brazil showed that EAEC markers were present in 7.1% of the *E. coli* isolates from UTIs (Abe et al., 2008). Neither of these studies identified clonal lineages of EAEC specifically associated with extra-intestinal infections. In 1991, an outbreak of UTIs (18 patients) occurred in Copenhagen caused by a multi-resistant *E. coli* O78:H10, confirmed to be an EAEC using molecular methods (Olesen et al., 2012). Genotyping by MLST of the outbreak isolates found all were ST10 and most carried a range of virulence genes including *fimH* (type 1 fimbriae; ubiquitous in *E. coli*); *fyuA*, *traT*, and *iutA* (associated with extra-intestinal pathogenic *E. coli*); and *sat*, *pic*, *aatA*, *aggR*, *aggA*, ORF61, *aaiC*, *aap*, and ORF3 (associated with EAEC). The source was never identified.

More recently, in a study of ESBL-producing *E. coli* from humans, foods and food-producing animals in three European countries (Wu et al., 2013), eight multidrug-resistant ESBL-producing EAEC were isolated from urine specimens, and one from a blood culture (Chattaway et al., 2014a). The multidrug-resistant EAEC isolates belonged to sequence type (ST) 38 and had various somatic antigens and $bla_{CTX\text{-}M}$ genes. ST38 is predominantly associated with UTIs, but MLST studies have demonstrated the organism's propensity to cause systemic infections. The authors of the study concluded that the ST38 strain identified had independently acquired the two phenotypes (UPEC and EAEC), which would suggest the emergence of a UPEC-EAEC mixed pathotype strain. Furthermore, on the basis of epidemiological, microbiological, and molecular characteristics, the authors have suggested that the UPEC-EAEC pathotype may be an evolving clonal group and that a single sequence type, ST38, which probably originated from the gut, is now associated with both multidrug resistance and with UTI (Chattaway et al., 2014a).

In a study of temporal trends within *E. coli* ST131:O25 and the H30 and H30-Rx sub-clones for AMR, virulence genes, biofilm formation, 12 (19%) non-H30 ST131 isolates (all from 1998 to 2004), no (0%) H30 sub-clone isolates fulfilled molecular criteria for EAEC ($P < 0.001$). Eleven of these isolates collected in the Copenhagen area from 1998 to 2000 were gentamicin-resistant. Of the 11, six were *pap*-positive urine isolates from patients with a UTI, and three were *pap*-negative (Olesen et al., 2014).

4.9. Stx-producing EAEC

The causative strain in the 2011 Stx-producing EAEC O104:H4 outbreak was unusual as it carried the EAEC genes *aggR*, *aggA*, *set1*, *pic* and *aap* as well as a prophage encoding the *stx*$_2$ gene (Bielaszewska et al., 2011). This outbreak brought to the attention of the scientific community the possibility that STEC may comprise elements of more than one single *E. coli* pathotype. Some authors have coined the term 'Enteroaggregative Haemorrhagic *E. coli*' (EAHEC) to describe these STEC strains (Brzuszkiewicz et al., 2011). The outbreak strain releases multiple virulence factors *via* outer membrane vesicles (OMVs) shed during multiplication. The OMVs contain Stx 2a, *Shigella* enterotoxin 1, H4 flagellin and O104 lipopolysaccharide. The OMVs bind to, and are internalised by, human intestinal epithelial cells *via* dynamin-dependent and Stx2a-independent endocytosis. They deliver the OMV-associated virulence factors intracellularly and induce caspase-9-mediated apoptosis and IL-8 secretion. Stx2a is the key OMV component responsible for cytotoxicity, and flagellin and lipopolysaccharide are the major IL-8 inducers. The OMVs represent novel ways for the *E. coli* O104:H4 outbreak strain to deliver pathogenic elements and damage host cells (Kunsmann et al., 2015).

It is important to consider that *E. coli* strains with the same virulence genes as Stx-producing EAEC O104:H4 have been previously observed on a few occasions. Such strains have been first reported as the causative agent of a small HUS outbreak that occurred in France at the beginning of the 1990s (Morabito et al., 1998). In that episode, the patients were infected with an *E. coli* O111:H2 strain showing the ability to adhere to cultured cells with the stacked-brick adhesion mechanism (Nataro and Kaper, 1998) and able to elaborate Stx2 (Morabito et al., 1998). Furthermore, a few sporadic cases of infection with Stx-producing EAEC strains of serotype O104:H4 were retrospectively described in the time period 2000–2010 soon after the German outbreak (Iyoda et al., 2000; Scavia et al., 2011). Finally, a sporadic HUS case caused by a Stx-producing EAEC O111:H21 in Northern Ireland in 2012 (Dallman et al., 2012) and a small outbreak of infection with a Stx–producing EAEC O127:H4 occurred and in Italy in 2013 (Tozzoli et al., 2014), respectively. Nowadays, four different serotypes of Stx-producing EAEC serotypes have been identified including O111:H2 (Morabito et al., 1998), O104:H4 (Bielaszewska et al., 2011), O111:H21 (Dallman et al., 2012) and O127:H4; this observation, together with the finding that the genomic backbone of Stx-producing EAEC is similar to that of non-Stx-producing EAEC, suggests that these strains could have emerged following the acquisition of an Stx-carrying phage from a bovine reservoir by an EAEC, and that this pathotype has undergone stabilization (Beutin et al., 2013).

From about 2,400 LEE-positive STEC isolates from cases of human infection referred to the German National Reference Centre between 2008 and 2012, two strains exhibited both EHEC and EAEC marker genes, and specifically were *stx2*- and *aatA*-positive. Like the 2011 outbreak O104:H4 isolates, one of these novel EHEC/EAEC strains harbored stx2a. This was isolated from a patient with bloody diarrhoea in 2010 and was serotyped as O59:H−, belonged to MLST ST1136, and exhibited genes for type IV aggregative adherence fimbriae (AAF). The second strain was isolated from a patient with diarrhoea in 2012, harbored stx2b, was typed as O rough:H−, and belonged to MLST ST26. Although this strain conferred the aggregative adherence phenotype, no known AAF genes corresponding to fimbrial types I to V were detected (Lang et al., 2015). A novel Stx-EAEC O59:NM (fliCH19 by molecular typing) strain has been isolated from cases of human infection in Argentina over an 11-year period. Eight of these were from cases of HUS and one from bloody diarrhoea. The strains were positive for *stx2a* and the *agg4A* fimbrial subunit genes, as in the German strain, but in addition, these nine strains were positive for *iha*, *lpf*O26, *lpf*O113, *aatA*, *aap, sigA* genes by PCR. By *Xba*I-PFGE the strains showed a high clonal relationship of more than 85% similarity (Carbonari et al., 2015; Lang et al., 2015).

These findings, plus the observation of some variants amongst the O104:H4 'outbreak' strains, suggest that the occurrence of mixed EAEC/STEC pathotype *E. coli* is likely to be an ongoing low frequency event and the occurrence of outbreaks probably relates primarily to opportunities for growth and dissemination of the organisms in foodstuffs or infected carriers (Prager et al., 2014; Fruth et al., 2015; Tietze et al., 2015).

4.10. Conclusions

- EAEC have been associated with travellers' diarrhoea and with acute diarrhoeal illness among children in both low-income and high-income regions, with severe diarrhoeal infections in immunocompromised patients and with UTI infections.
- The clinical manifestations of EAEC infection vary from individual to individual, depending upon the genetic composition of the host and of the strain. Infections with EAEC may be asymptomatic, with persons carrying such strains exhibiting no overt disease symptoms.
- A predominant feature of EAEC infection in low-income countries is the propensity to cause persistent diarrhoea for more than two weeks, making these bacteria a significant cause of mortality, particularly in children.
- Stx-producing EAEC, such as the 2011 *E. coli* O104:H4 outbreak strain, have been associated with a range of symptoms such as HUS and HC, and have resulted in deaths in infected individuals.
- The emergence of mixed EAEC/STEC pathotype *E. coli* is likely to be an ongoing low frequency event and the occurrence of outbreaks probably relates primarily to opportunities for growth and dissemination of the organism(s) in foodstuffs or infected carriers.
- Strains of the EAEC pathotype are considered to be adapted to the human host. There is no evidence for animals being a reservoir of EAEC in EU MSs. Putative carriage of EAEC by animals is often based on the reporting of genes such as *astA*, which are not specific for EAEC, in parts of the world where pollution by human faecal waste is common.
- Outbreaks of EAEC associated with foods are frequently suggestive of contamination by asymptomatic food handlers and to poor sanitation.
- Multiple pathogen outbreaks in which a range of pathogens as well as EAEC are implicated are being increasingly identified.
- Contamination of the environment, particularly watercourses, can occur in parts of the world where human sanitary systems are insufficient and EAEC is prominent in people. Environmental contamination may also be a pathway for EAEC occurrence on produce.
- Prolonged survival of EAEC for at least several weeks in wet and dry substrates appears to be possible, but further controlled studies under laboratory and natural conditions are needed to fully quantify this.
- Biofilm formation amongst EAEC strains is variable and involves several gene combinations. The ability to form biofilms is linked to the severity of human disease and is likely to be involved in survival in the environment.

5. Microbiological methods for the detection, identification and characterisation of EAEC (ToR 3)

Testing of food and faecal samples involves the detection of EAEC-associated traits in the matrix or in enrichment culture from such matrices, followed by isolation of the organism and confirmation of the presence of EAEC-associated genes or phenotyes using molecular- and culture-based techniques, as described below.

5.1. Detection of EAEC in food by Real Time PCR amplification of the *aggR* and *aaiC* genes

Following the Stx-producing *E. coli* O104:H4 outbreak in 2011, the EU RL VTEC has developed a molecular methodology to screen food samples for the presence of EAEC by the detection of targets designed on the *aggR* and *aaiC* genes The same genetic markers have been indicated by ECDC and EFSA for the identification of EAEC strains. The protocol includes an enrichment method for the screening of food samples and conditions for the Real Time PCR amplification of the *aggR* and *aaiC* genes, and also the DNA sequence and characteristics of the primers and probes used for the detection of EAEC.

In the Opinion of the WG this protocol, included in Appendix G, is presently considered a good candidate for adoption as the preferred method for the molecular detection of EAEC in food matrices by EU MSs.

5.2. Adhesion tests

At the end of the 1980s, the enteroaggregative adhesion was identified as a peculiar diffuse adhesion pattern that some *E. coli* strains produced onto Hep-2 cells monolayers, resembling the position of the brick in a wall and defined as 'stacked brick' (Nataro et al., 1987). Since then, adhesion tests on monolayers of HEp-2 and HeLa cells have been considered the gold standard assays for the identification of the EAEC (Haider et al., 1992). Although allowing for sensitive identification of this *E. coli* pathogroup, the adhesion assays are cumbersome and require experienced personnel and specialised facilities, and can therefore only be conducted in reference laboratories, making them not suitable for a routine testing of samples from human infections.

Molecular biology has largely superseded the phenotypic adhesion assay and provides a way to simplify the screening for EAEC. Since the initial characterisation of EAEC, the aggregative adhesion properties appeared to be associated with the presence of a 55–65 megadalton (Mda) plasmid (Vial et al., 1988) and the design of molecular screening tools was directed towards the use of sequences from this plasmid. Baudry and colleagues developed a DNA probe, CVD432, which showed a high degree of correlation with the phenotypic assay (Baudry et al., 1990). However, a number of subsequent studies conducted using the CVD432 probe for screening EAEC strains isolated from cases of diarrhoea in different geographic locations showed more variable results (Okeke and Nataro, 2001).

5.3. PCR-based methods

In 1995 the first PCR tool was developed based on the sequence of the *Eco*RI/*Pst*I fragment of pCVD432 plasmid, corresponding to the CVD432 probe (Schmidt et al., 1995). Such a PCR was evaluated against the cell culture adhesion and the hybridization assays and returned a 97.7% concordance. In detail, most of the 456 *E. coli* strains negative to the adhesion assay included in the experimental design were negative to both the PCR and hybridization, while five of the seven strains displaying the aggregative adhesion pattern onto Hep-2 cells monolayers were positive to both the molecular assays. Finally, a few strains negative by the adhesion test were positive when subjected to the molecular approach, lowering its overall specificity (Schmidt et al., 1995). An additional PCR primers pair was designed on the sequence of the CVD432 probe sequence and included into a multiplex assay for the detection of diarrhoeagenic *E. coli* together with primers specific for the *eae*, *stx*, *st*, *lt*, *ipaH* genes (Toma et al., 2003). The multiplex assay was evaluated using 30 EAEC confirmed strains and proved 100% accurate in terms of correctly identifying strains (Toma et al., 2003). Similar findings were reported for the same EAEC primers used in multiplex assays designed in other studies using either artificially contaminated stool samples (Aranda et al., 2007) or reference strains collection (Brandal et al., 2007).

The PCR tool based on the CVD432 probe sequence deployed by Schmidt et al. (1995) was used in a study aiming at assessing the importance of EAEC as aetiological agent of acute diarrhoea among children in Calcutta, India, in parallel with the adhesion test conducted with the HeLa cultured cells (Dutta et al., 1999). A blind comparison of the two methods was done using the *E. coli* strains isolated from 254 children with acute diarrhoea and returned a sensitivity value for the PCR of 78.8% and a specificity of 97.5% when the adhesion test was used as the gold standard assay (Dutta et al., 1999). The limited correlation of the molecular hybridization and PCR assays suggested that, in spite of the initial strong association of the presence of the plasmid with the ability to induce the stacked brick pattern of adhesion, there was a certain degree of variability in the plasmid structure and therefore studies aiming at a more complete characterization of the plasmid itself and assays based on the detection of more than one marker have been deployed. In 2003, EAEC isolates displaying the enteroaggregative pattern of adhesion on cultured HeLa cells were screened by PCR for the presence of a panel of genes present on the large plasmid pCVD432 (Tsai et al., 2003). Specific primers were designed on the sequences of the genes *astA*, *aafA* and *aggA* (Tsai et al., 2003) and used for the screening in addition to the CVD432 primers deployed by Schmidt et al. (1995). The study included six *E. coli* strains with the typical stacked brick adhesion and about 400 negative strains counting either laboratory strains (60 isolates) or isolates from the Taichung Veterans General Hospital, Taichung,

Taiwan (337 isolates). The study showed that the primers deployed on the CVD432 probe correctly identified all the six EAEC similarly to the primers pair for the *aggA* gene, while those amplifying the *aaf* and the EAST1-encoding gene gave positive results for one and four of the six EAEC isolates respectively (Tsai et al., 2003).

Another multiplex PCR, based on the detection of *aat* (CVD432 probe), *aaiA* and *astA* genes sequences, has been explored as a means for rapid and accurate identification of EAEC infection (Jenkins et al., 2006b). There is no consensus on a single PCR assay to be used in the routine testing of EAEC isolates. Nevertheless, the most recent approaches attempting to standardize EAEC identification revolve around the possibility to use PCR amplification of markers from the plasmid and the chromosome such as the proposed association between the genes *aaiA*, *aaiG*, *aggR* and *aatA* (Andrade et al., 2014) or *aaiC* and *aggR* (Scheutz et al., 2011; EFSA BIOHAZ Panel, 2013).

The attempt to identify markers that could serve as good candidates for a diagnostic assay and be associated with the virulent EAEC led to the publication of a number of studies making use of a diverse range of genes located on the large plasmid pCVD432. Cerna et al. (2003) developed a multiplex PCR assay based on the amplification of the genes *aap*, and *aggR*, and of the plasmid fragment corresponding to the CVD432 probe. The proposed multiplex approach has demonstrated that 82% of the EAEC strains isolated from patients with diarrhoea (23 out of 28) were positive for the three loci simultaneously. The three loci were reported to be present in a vast proportion of the strains assayed in other studies (Czeczulin et al., 1999), and it has been suggested that they may be phylogenetically or pathogenically linked.

Given the recognized heterogeneity of EAEC, other studies focussed on the identification of markers associated with the virulence of these *E. coli* strains. Muller et al. (2007) chose a PCR approach based on the detection of the plasmidic genes *astA*, *aggR*, and *pic* for EAEC identification embedded in a multiplex PCR assay including thirteen genetic determinants for the identification of all the diarrhoeagenic *E. coli*. The assay responded proficiently with all the strains tested, but highlighted once again the great diversity of the EAEC, as only 85% of the strains identified as EAEC by PCR actually induced the stacked-brick aggregative-adherence pattern in tissue cultures, while the strains negative in the gold standard assay were still positive for *astA* and/or *pic* genes (Muller et al., 2007).

In many cases most of the plasmid markers used in all the PCR assays described seem to be adequate to correctly identify EAEC (Schmidt et al., 1995; Czeczulin et al., 1999; Dutta et al., 1999; Sheikh et al., 2002; Tsai et al., 2003; Aranda et al., 2007; Brandal et al., 2007; Muller et al., 2007; Cordeiro et al., 2008; Gomez-Duarte et al., 2009; Baranzoni et al., 2014). The variability of the plasmid structure and sequence, and the possibility that this mobile genetic element may be lost has led to the conclusion that chromosomal markers had to be included in the molecular screening assays. Extensive genotyping of EAEC was performed in different studies (Jenkins et al., 2006a; Boisen et al., 2012) but it was recognized that, similarly to the plasmid-associated genes, no chromosomal markers are present in 100% of EAEC or are associated with EAEC. Some markers have been identified as being significantly associated with those EAEC isolated from clinical illness such as the *Shigella* SPATE toxin SepA (Boisen et al., 2012).

5.4. Proficiency tests

In the EU, three proficiency tests (PT) involving EAEC strains in their scope have been organised by the EU RL VTEC for the benefit of the network of NRLs for *E. coli.* The PT10, PT11 and PT13 were organised in 2012, 2013 and 2014, respectively, and aimed at assessing the preparedness of the EU NRLs towards the identification of EAEC isolates.[6] In the three PT rounds the laboratories were asked to use the method provided by the EU RL VTEC to identify isolated *E. coli* strains as EAEC by means of real time PCR amplification of *aagR* and *aaiC* genes.[7] Thirty-four, 32 and 40 NRLs, respectively, participated in the three studies with performances ranging from 91% (PT10) to 96.8% (PT11) and 100% (PT13) success in the ability of NRLs in identifying the EAEC test strain.

The ECDC FWD me has supported external quality assessment (EQA) schemes specifically organised for NRLs participating in the European FWD network. To test the laboratories for the capacity to detect the virulence characteristics related to enteroaggregative isolates a selected EAEC strain with

[6] Reports available at: http://www.iss.it/vtec/index.php?lang=2&anno=2015&tipo=15
[7] Reports available at: http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_05_Rev_1.pdf

aggR and *aaiC* virulence genes were included in the EQA scheme for typing of verocytotoxin/Stx-producing *E. coli* (STEC) in 2013 (EQA-4) and 2014 (EQA-5). From 20 and 22 laboratories participating in the two EQAs, respectively, 11/17 laboratories (39%/61%) reported results for EAEC virulence genes (*aggR* and *aaiC*) in the EQA-4 and 20/16 laboratories (69%/55%) in the EQA-5. All laboratories correctly detected *aggR* and *aaiC* genes of the test strain.[8]

5.5. Whole Genome Sequencing (WGS)

Whole genome sequencing (WGS) can provide information on the population structure of EAEC using a whole genome MLST (wgMLST) approach or the analysis of SNPs. The pAA is regarded as a defining feature of EAEC but recent WGS analysis has shown that pAA is associated with a wide range of plasmid replicon types and that it has a diverse genomic architecture (Dallman et al., 2014).

WGS can determine the presence or absence of all the major putative EAEC virulence genes, including *aggR, aat, aap, sepA, sigA, pic*, AAF types I-V and, more recently, a putative isopentyenyl isomerise (IDI) enzyme (Rasko et al., 2011). WGS data have also been used to determine the integrity of the chromosomally-encoded AAI operon and to provide information on AMR (Dallman et al., 2014).

To our knowledge WGS has not been used routinely for the detection of EAEC either from cases of human infection or from foods. As technology is progressing rapidly, further studies will be needed to assess the potential of WGS for such purposes.

5.6. Conclusions

- Testing of both food and faecal samples involves the detection of EAEC-associated traits in the matrix or in enrichment culture from such matrices by molecular methods, followed by isolation of the organism and confirmation of the presence of EAEC-associated genes or phenotypes.
- The most widely-recognised diagnostic option for EAEC isolates from a technical point of view remains the adhesion assay onto monolayers of cultured epithelial cells. This approach is cumbersome, expensive and requires experienced personnel and is therefore confined to the reference laboratories.
- The widespread use of PCR combined with the increased availability of information on the virulence gene asset of EAEC has led to the development of a number of gene-based assays that have performed well. Such assays have been effectively used for the confirmation of EAEC infections.
- There is no consensus on a single PCR assay to be used in the routine testing of EAEC. The most recent approaches attempting to standardize EAEC identification revolve around the use of PCR amplification of specific markers from the plasmid and the chromosome.
- PCR-based methods can be used for the analyses of food for the presence of EAEC. In this respect the protocol developed by the EU RL VTEC for the detection of EAEC in food by Real Time PCR amplification of the *aggR* and *aaiC* genes and used in the EU RL VTEC Proficiency Testing schemes is presently considered a good candidate for the molecular detection of EAEC in food matrices by EU MSs.
- Whole genome sequencing (WGS) can provide information on the population structure of EAEC using a whole genome MLST (wgMLST) approach or the analysis of single nucleotide polymorphisms (SNPs). WGS can also determine the presence or absence of all the major putative EAEC virulence genes.
- WGS has not been used routinely for the identification of EAEC from either cases of human infection or from foods. Further studies are required to assess the potential of WGS for such purposes.

[8] Reports available at: http://ecdc.europa.eu/en/publications/Publications/4th-External-Quality-Assessment-typing-of-verocytotoxin-producing-E.-coli-VTEC-web.pdf and http://ecdc.europa.eu/en/publications/Publications/VTEC-EQA-2014.pdf

6. Nature, extent and public health significance of antimicrobial resistance in EAEC

6.1. Isolates from patients

Although most cases of bacterial enteritis are self-limiting, acute or persistent infections may require the use of antibiotic treatment, which may be compromised if resistance is present (Kong et al., 2015). Data on AMR in EAEC are sporadic, but available information is summarised below:

6.1.1. Sporadic infections

In Africa, EAEC serotype O44 with resistance to tetracyclines, ampicillin, erythromycin, trimethoprim-sulphamethoxazole, and amoxicillin/clavulanate, and susceptibility to chloramphenicol, nalidixic acid, azithromycin, and cefuroxime was reported in children in Kenya, with persistent diarrhoea between 1991 and 1993 (Sang et al., 1997). The widespread use of ampicillin, tetracycline and trimethoprim–sulphamethoxazole was thought to be a major factor contributing to the emergence of such resistant strains. More recently, in 2003–2004, EAEC isolates with a high incidence of resistance to cotrimoxazole, ampicillin and tetracyclines were observed in food handlers in that country (Oundo et al., 2008). In south-east Asia, EAEC isolates in Thailand in 1992 were reported to be resistant to several antibiotics routinely used for the treatment of gastroenteritis in that country, including co-trimoxazole and amoxicillin (Yamamoto et al., 1992).

In a study in southeast China from 2009 to 2011, Chen et al. investigated 347 isolates of diarrhoeagenic *E. coli*, of which 217 (62.5%) were EAEC. Over 70% of isolates exhibited multiple drug resistance and over 90% of isolates were resistant to ampicillin (Chen et al., 2014).

In the Indian sub-continent, among 64 EAEC strains isolated in southern India from children and adults with diarrhoea from 2006 to 2007, disk diffusion testing for 11 commonly used antimicrobial agents showed EAEC resistance to co-trimoxazole, ampicillin and nalidixic acid in the majority of isolates, with 75% of isolates exhibiting multidrug resistance. Most of the isolates showing multidrug resistance were from children below 5 years of age, and an increase in isolates with resistance to quinolones was observed over the period of study (Raju and Ballal, 2009). Resistance to ampicillin, cefotaxime, gentamicin, co-trimoxazole, nalidixic acid and ciprofloxacin has been reported in EAEC isolates from travellers from India returning to Spain, with resistance to cefotaxime in such isolates encoded by a CTX-M-15 β-lactamase (Guiral et al., 2011), and with resistance to nalidixic acid linked to mutations in the *gyrA* gene alone or in both *gyrA* and *parC* genes (Vila et al., 2001). In studies in Central and South America, in a prospective passive diarrhoea surveillance cohort study of 1,034 infants of low socioeconomic communities in Lima, Peru, from 2006 to 2007, the most common *E. coli* pathogens in cases of diarrhoea were EAEC (14%), of which greater than 90% of isolates were resistant to antimicrobials, with resistance to ampicillin (91%), co-trimoxazole (85%) tetracyclines (73%), and nalidixic acid (33%) predominating; in this study, 70% of isolates were multiresistant to three or more antimicrobials (Ochoa et al., 2009). In a study of children with and without diarrhoea in Nicaragua in 2005 and 2006, EAEC isolates exhibited significantly higher levels of resistance to ampicillin and co-trimoxazole compared to the other diarrhoeagenic *E. coli* categories (Amaya et al., 2011).

In a study of 456 enteropathogens from cases of travellers' diarrhoea returning to the USA from Mexico, India, and Guatemala between 2006 and 2008 to determine changes in susceptibility against 10 different antimicrobials by the agar dilution method in comparison with an earlier study performed in 1997, traditional antibiotics, such as ampicillin, co-trimoxazole, and doxycycline, isolates continued to show high levels of resistance. EAEC isolates from Central America showed increased resistance to nearly all of the antibiotics tested. Compared to minimum inhibitory concentrations (MICs) of isolates made 10 years prior, there were 4- to 10-fold increases in MIC(90) values for ceftriaxone, ciprofloxacin, levofloxacin and azithromycin (Ouyang-Latimer et al., 2010).

In Europe, of 160 strains of *E. coli* identified as EAEC which had been isolated from patients in the UK with infectious intestinal disease or gastro-enteritis between 1993 and 1996, over 50% were resistant to one or more of eight antimicrobials, and 30 (19%) were resistant to four or more drugs with one strain being resistant to eight antimicrobials (Wilson et al., 2001), In Poland, EAEC isolates in the stools of children with diarrhoea in 2003 were resistant to ampicillin, tetracyclines, trimethoprim,

sulfamethoxazole, and chloramphenicol (Sobieszczanska et al., 2003). In Spain, in studies of EAEC isolates from patients presenting with traveller's diarrhoea in the periods 1994–1997 and 2001–2004, the highest levels of resistance in EAEC were found for tetracyclines (70%), ampicillin (57%) and cortrimoxazole (52%), followed by chloramphenicol (37%), nalidixic acid (12%), amoxicillin plus clavulanic acid (10.5%) and ciprofloxacin (3%). A statistically significant increase in resistance to chloramphenicol and amoxicillin plus clavulanic acid was observed in EAEC when the two periods were compared (Mendez Arancibia et al., 2009).

Elsewhere, in a study in Iran from 2007 to 2008 of 140 children with diarrhoea, 15 (10.7%) EAEC strains were identified of which 100% exhibited resistance to ampicillin, 100% to erythromycin, 79% to cephalothin, 71% to co-trimoxazole, 64% to tetracyclines, and 57% to nalidixic acid, with 43% exhibiting reduced susceptibility to resistance to ciprofloxacin (Aslani et al., 2011). In a more recent limited study of DAEC from adolescents and adults in that country, all eight EAEC isolates detected were resistant to at least one antimicrobial, with resistance to ampicillin, tetracyclines and nalidixic acid (Alikhani et al., 2013).

6.1.2. Outbreak-associated infections

Urinary tract infection of multiresistant *E. coli* O78:H10; Denmark, 1991

In 1991, a multiresistant *E. coli* O78:H10 strain identified by molecular methods as EAEC caused an outbreak of UTIs in Copenhagen, Denmark. All isolates from the outbreak exhibited the same distinctive resistance profile, i.e. ACSSuTTp (ampicillin, chloramphenicol, streptomycin, sulphonamides, tetracyclines, and trimethoprim) (Olesen et al., 2012). The source of the outbreak strain was not identified, but there was no evidence of food involvement.

Shiga toxin (Stx)-producing EAEC O104:H4 outbreak; EU, USA and Canada, 2011

The 2011 O104:H4 outbreak strain exhibited resistance to a wide range of β-lactamase antimicrobials including ampicillin, amoxicillin/clavulanic acid, piperacillin/sulbactam, piperacillin/tazobactam, cefuroxime, cefotaxime, cefpirome and cetfazidime, and was also resistant to streptomycin, nalidixic acid, tetracyclines and trimethoprim and the sulphonamides, but was susceptible to the carbapenems (Bielaszewska et al., 2011; EFSA, 2011a; Rasko et al., 2011; Scheutz et al., 2011). The strain contained an 88.5-kb IncI1-ST31 plasmid –pESBL-EA11- that encoded bla-CTX-M-15 and bla-TEM, (Yamaichi et al., 2014). Although not considered important in the treatment of affected persons in this outbreak, the presence of resistance genes may have contributed to the development and spread of the causative organism.

During screening of people at risk of acquisition of the *E. coli* O104:H4 outbreak strain, another ESBL-producing and Shiga toxin-positive *E. coli* of serotype O91:H14 was identified from the faeces of a human patient. The patient also carried a further ESBL-producing but Stx-negative *E. coli* strain. Both isolates harboured *bla*-CTX-M-15 and *bla*-TEM-1 on an IncI1-ST31 plasmid, which was indistinguishable in terms of size and plasmid restriction pattern to the plasmid of the epidemic *E. coli* O104:H4 strain. The patient had travelled to India 6 months prior to the isolation of the *E. coli* strains (Arvand et al., 2015). This suggests the international dissemination of bacteria such as EAEC and highlights the involvement of mobile genetic elements (e.g. plasmids) and phage in the creation of potentially epidemic multi-drug resistant STEC strains.

Multi-pathogen foodborne outbreak, UK, 2013

Ten EAEC serotypes were identified in faecal samples recovered from patients in the large and complex multi-pathogen foodborne outbreak in the UK in February/March 2013 (see Section 3.2.3 above). Of 20 EAEC isolates characterised, several resistance profiles were identified, ranging from nalidixic acid alone through to ampicillin, sulphonamides, streptomycin, nalidixic acid, ceftazidine, cefataxime, ceftiofur and cefpirome (Dallman et al., 2014).

6.2. Isolates from food-producing animals

In a study conducted in 2004 on a large number of cattle, sheep and pigs at slaughter in the UK no EAEC was detected in the intestinal content of the animals (Cassar et al., 2004). There have been no

reports of EAEC and consequently of AMR in EAEC amongst isolates of *E. coli* from food-producing animals in any further studies in EU countries, although a small number of EAEC-like isolates with resistance to nalidixic acid, amoxicillin, amoxicillin-clavulanate, streptomycin, tetracyclines, imipenem or combinations of these resistances have been identified in a 2012 Brazilian study of avian organic fertilizers (Puno-Sarmiento et al., 2014).

6.3. Isolates from foods

To our knowledge the only report of AMR in an EAEC isolate from food was in the study of Zurfluh et al. (2015) (see Section 4.2 above) in which an isolate made from a vegetable (okra) imported into Switzerland from India exhibited resistance to ampicillin and third-generation cephalosporins through possession of a CTX-M-14 cephalosporin-inactivating enzyme. Although not stated in the publication, the strain was most likely to have been of human origin.

Several food-related EAEC outbreaks have been described in which strains from patients were resistant to at least one antimicrobial. For example, in one outbreak of *E. coli* O untypeable: H10 in Japan in 1993 associated with school lunches, in which over 2,600 children were affected, all isolates were susceptible to nalidixic acid, chloramphenicol, streptomycin, kanamycin, and cephalothin, but were resistant to ampicillin (Itoh et al., 1997). In an outbreak of GI illness in April 1991 among patrons of a restaurant in the USA and in which a strain of *E. coli* O39:NM exhibiting properties of EAEC was isolated from affected individuals, the outbreak strain was resistant to tetracyclines, ampicillin, and carbenicillin and carried four plasmids with molecular weights of 55, 45, 5.0 and 2.6 Mda (Hedberg et al., 1997). In the 2011 Stx-producing *E. coli* O104:H4 outbreak, fenugreek seeds were epidemiologically-implicated as the vehicle of infection (see Section 4.2 above). The outbreak strain exhibited resistance to ampicillin, amoxicillin/clavulanic acid, cefotaxime, streptomycin, nalidixic acid, tetracyclines, trimethoprim and sulphonamides (Buchholz et al., 2011). In the multi-pathogen foodborne outbreak in the UK in 2013 discussed above (see Section 4.3), a wide range of resistance profiles ranging from nalidixic acid alone through to ampicillin, sulphonamides, streptomycin, nalidixic acid, ceftazidine, cefataxime, ceftiofur and cefpirome were identified in EAEC isolates from patients (Dallman et al., 2014), but such isolates were not obtained from foods implicated in the outbreak.

6.4. Importance to public health of antimicrobial resistance in EAEC

6.4.1. Implications for therapy

Many EAEC infections are self-limiting and in such cases antibiotic therapy is not required (Huang and Dupont, 2004). Nevertheless, aggressive and chronic cases of infectious diarrhoea often require therapeutic interventions to ensure resolution and halt progression to dehydration, hypovolaemic shock and death. When antibiotic therapy is considered necessary, antimicrobial treatment should be individual-based (Croxen et al., 2013). The most frequently used first-line antimicrobials which have traditionally been used for the treatment of travellers' diarrhoea are ampicillin, co-trimoxazole, tetracyclines (doxycycline) and quinolones, due to their ready availability and inexpensive cost (Ouyang-Latimer et al., 2010; Kong et al., 2015), and ampicillin and co-trimoxazole have been recommended for this purpose by the World Health Organization (Aslani et al., 2011). In clinical trials ciprofloxacin and rifaximin compared to placebo have been shown to significantly shorten the course of diarrhoea in patients who developed EAEC infection (Huang et al., 2004).

As EAEC have become increasingly resistant to various antibiotics, selection of an appropriate antibiotic should take into account the region of the world where the infection was acquired, as there are different antimicrobial susceptibility patterns for many geographical regions (see above). Ideally, EAEC isolates should be subjected to antimicrobial susceptibility testing before treatment commences, although this may not be feasible in low-income countries.

Like *Shigella* infections, EAEC infections have been successfully treated with ciprofloxacin and other fluoroquinolones, although this group of antimicrobials are not in general regarded as suitable for use in children; cephalosporins, rifaximin and azithromycin have been used with some success. In adult patients in the USA EAEC have been shown to be susceptible to rifaximin (Infante et al., 2004) or to single dose of azithromycin with or without loperamide (Ouyang-Latimer et al., 2010). The emergence of multiple antimicrobial-resistant strains often coupled with resistance to quinolones and third-

generation cephalosporins has compromised treatment in some regions (Kong et al., 2015) and in returning travellers, particularly from India and Mexico (Vila et al., 2001; Guiral et al., 2011).

In treatment of infections caused by Stx-producing EAEC strains, because of the risk of promoting the development of HUS by stimulating Stx production by the use of the usual antibiotics, such as ciprofloxacin, clinicians have selected azithromycin, which *in vitro* represses *stx* expression (Croxen et al., 2013). Nonetheless, the use of azithromycin to eliminate carriage of Stx-producing strains from patients or food handlers is still considered a controversial treatment (Seifert and Tarr, 2012).

In relation to the public health relevance of AMR in EAEC, such organisms frequently exhibit resistance to one or more of the antimicrobials commonly used in human medicine, including antimicrobials regarded as critically-important for human health. As such, the use of antimicrobials will enhance the survival and spread of EAEC in ecosystems where such agents are deployed.

6.4.2. Comparison with zoonotic *E. coli* pathotypes (e.g. STEC)

Of note for EAEC is the high occurrence of AMR in comparison to other *E. coli* pathotypes associated with food production animals, such as STEC. Although AMR has been identified in STEC from both human infections (Threlfall et al., 1999; Roest et al., 2007; Buvens et al., 2012) and from cattle and beef products (Ennis et al., 2012; Sasaki et al., 2012), resistance does appear to be less common than in EAEC isolates from cases of human infection. For example, for isolates of STEC from cases of infection in the UK from 1996 to 1999, less than 20% exhibited resistance to antimicrobials, with isolates with resistance to streptomycin, sulphonamides and tetracyclines predominating and with no isolates exhibiting multiple resistance (to four drugs or more) (Threlfall et al., 1999). Similarly, in isolates of STEC from cases of infection in the Netherlands between 1998 and 2003, only 29% of isolates strains were resistant, of which less than 50% were resistant to three or more antimicrobials (Roest et al., 2007). From cattle in the Republic of Ireland, AMR was observed in only 29% of 84 isolates of 33 different STEC serotypes from cattle in a survey reported in 2012, with less than 50% of such isolates exhibiting multiple resistance (Ennis et al., 2012). In a study of STEC from cattle in Japan reported in 2012, resistance to one or more antimicrobial agents was detected in only 32 of 241 (13%) of the STEC O157 isolates.

The figures for AMR in STEC isolations from cases of human infection and from cattle should be contrasted with those for EAEC isolations described above, where the occurrence of resistance to at least one antimicrobial was frequently above 50%, rising to 100% in some investigations, and with multiple resistance often the norm. Possible explanations for this anomaly may be related to either differences in the innate propensity of STEC and EAEC strains to acquire and maintain plasmids encoding for AMR, or to antimicrobial selective pressure, with patients with EAEC infections more likely to have been exposed to antimicrobials than cattle, which are acknowledged as a major reservoir of STEC.

6.5. Conclusions

- AMR is increasingly reported on a world-wide basis in isolates of EAEC in cases of sporadic diarrhoea in children and adults in both low-income and high-income countries, in returning travellers, and in outbreaks of infection.
- Antimicrobials such as ampicillin, co-trimoxazole and doxycycline have been used to treat EAEC infections. Fluoroquinolones and third-generation cephalosporins have been increasingly used, but the emergence of multiple antibiotic-resistant strains often coupled with resistance to quinolones and third-generation cephalosporins has compromised treatment.
- Food-borne outbreaks of EAEC in which the causative strains have exhibited antimicrobial resistance have been reported in recent years. The origin of the resistance genes in such strains has not been conclusively established.
- EAEC are more likely to exhibit resistance to antimicrobials than other pathogenic *E. coli* such as STEC. Such differences may be strain-related or associated with differences in antimicrobial selective pressure.

- EAEC frequently exhibit resistance to one or more of the antimicrobials commonly used in human medicine. As such, the use of aforesaid antimicrobials will enhance their survival and spread in ecosystems where such agents are deployed.

7. Research and surveillance needs (ToR 5)

When assessing the public health relevance of EAEC in general, the importance of AMR in EAEC, and the role of foods in EAEC outbreaks in EU MSs it is important to understand that, in light of the lack of definitive quantitative information, any discussion on 'relevance to public health' is subjective. To assist in providing more definitive information on the public health relevance of EAEC in EU MSs and the role of foods in EAEC outbreaks, the following research and surveillance needs have been identified:

7.1. Research needs

PCR-based methods for the detection and quantification of EAEC in food items should be further developed, validated and made available, as this would assist the assessment of human exposure, e.g. in a foodborne outbreak. Similarly, a standardised and validated multiplex approach to identification of the causal agents of diarrhoeal illnesses that involve multiple pathogens is required.

Adaptation of such methods for animal samples should be considered.

As *E. coli* strains possessing new combinations of virulence factors and AMR genes are emerging in foodborne outbreaks and infections, identification of drivers for incorporation of, e.g. *stx* genes into EAEC strains and the resultant fitness cost/benefits to strains is an important research requirement.

To better understand the routes by which humans are exposedto EAEC, methods for typing and comparative analysis should be further developed and applied to isolates collected from all potential reservoirs.

The use of culture media containing customised combinations of relevant antimicrobials to enhance detection of multiple drug-resistant EAEC amongst mixed populations of organisms should be investigated and optimised.

In order to gain a quantitative assessment of the occurrence of AMR in EAEC and the primary sources of resistance genes in EAEC isolates, including EAEC isolates from, e.g. UTIs, a more definitive statistically-based study of AMR in pathogenic *E. coli* serogroups principally associated with human infections, in comparison with those associated with foods and other possible sources, should be undertaken

Prolonged survival of EAEC for at least several weeks in wet and dry substrates appears to be possible, but further controlled studies under laboratory and natural conditions are needed to fully quantify the population dynamics of EAEC strains.

Biofilm formation amongst EAEC strains is variable and involves several gene combinations. The ability to form biofilms is linked to the severity of human disease and is likely to be involved in survival in the environment and reduced activity of biocides used on fresh produce. More research is needed to elucidate the mechanism of EAEC attachment and survival on fresh produce and seeds.

Improved understanding of the characteristics of EAEC strains in different environments, especially factors influencing their survival, multiplication and ability to uptake new virulence genes, is required.

The possible role of immunologic and genetic host factors in the response to EAEC infection needs to be studied in more detail.

To our knowledge WGS has not been used routinely for the identification of EAEC either from cases of human infection or from foods. As technology is progressing rapidly, further studies will be needed to assess the potential of WGS for such purposes

7.2. Surveillance needs

Because of the increasing evidence of the involvement of EAEC in severe disease, surveillance of EAEC in human diarrhoeal infection should be improved.

As data are sparse, monitoring programmes or surveys should also cover areas such as carrier status of humans, particularly food handlers and animals; and occurrence in foods (including foods of non-animal origin) and the environment, in order to get a better picture.

The evidence for animals and/or food being an important source of EAEC is sparse and is often based on the reporting of genes such as *astA*, which are not specific for EAEC, or in studies performed in parts of the world where pollution by human faecal waste is common. To quantify the possible involvement in EU MSs of EAEC strains in foods originating from such countries, a survey of imported non-EU foods should be considered.

In view of the potential for EAEC to provide an enhanced platform for attachment of highly pathogenic *E. coli* strains to the human intestine and because prevalence data in food animals are sparse, testing for EAEC genes in several possible reservoirs is needed. Investigations in animal intestinal samples that are already routinely selected for the harmonised monitoring of antimicrobial resistance in the EU would provide data for risk assessment. As regards the main livestock animal species (cattle, pigs, poultry) and the products derived from them, use could be made from the samples taken in the context of the implementation of Commission Decision 2013/652/EC.[9] There, caecal contents samples taken for detection of ESBLs at slaughterhouses and meat samples taken at retail could be used to test specifically for EAEC genes using a standardised and validated PCR-based protocol. Isolation of EAEC for further characterisation could then focus on gene positive samples. Such a study could provide statistically valid and representative data from the whole EU to confirm the expected absence of EAEC in the primary food chain.

For other possible vehicles, such as vegetables, herbs and fruits (especially berries), specific sampling and testing for EAEC needs to be undertaken to get an overview on the possible presence of EAEC genes in these types of products. Imported products should be seen as separate from those produced within the EU, and country-specific results would be desirable (assuming that the potential for EAEC contamination differs between countries).

In case of food- and water-borne outbreaks of diarrhoeal disease with unknown etiology, the inclusion of EAEC in the screening of infected individuals and foods should be encouraged.

A standardised and validated gene-based protocol should be utilised to explore samples from the main livestock animal species (cattle, pigs, poultry) and the products derived from them for the presence of EAEC and zoonotic *E. coli*. Similarly, sampling protocols for vegetables, herbs and fruits should be developed and applied accordingly.

Spread of EAEC in the environment may be difficult to assess. As soon as findings in animals, food or in humans are reported in a country, more detailed investigations should be started. Environmental studies may include waste water treatment plants, irrigation water and effluents from food processing plants and hospitals, as well as the biosolids derived from compost and anaerobic digestion of sludge used as top soil improvers.

Reporting should be adjusted to encourage MSs to provide specifically the information as to whether they have searched for EAEC in food and animals and, if so, the outcome of such searches.

Additionally, to assess the importance of the EAEC-associated *aggR* and *aaiC* genes genes in STEC strains, MSs should be further encouraged to report the occurrence of such genes together with the reporting of the STEC strains, as defined in the EU case definition (Commission Decision 2012/506/EU[10]).

In light of the apparently increasing involvement of EAEC in mixed pathogen outbreaks, surveillance of foods associated with such outbreaks for EAEC should be encouraged.

To better understand the pathways EAEC strains take to cause infections, isolates should be collected from different potential reservoirs and comparatively analysed.

[9] Commission Implementing Decision 2013/653/EU of 12 November 2013 as regards a Union financial aid towards a coordinated control plan for antimicrobial resistance monitoring in zoonotic agents in 2014. OJ L 303, 14.11.2003, p. 40–47.

[10] Commission Implementing Decision 2012/652/EU of 8 August 2012 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council. OJ L 262, 27.7.2012, p. 1–57.

PCR-based methods for detection and quantification of EAEC in food items should be made available, as this would assist the exposure assessment of persons at risk, e.g. in a foodborne outbreak.

7.3. Conclusions

A range of research and surveillance requirements have been suggested, which are targeted at assisting in the identification of EAEC in food-related infections in foods and in food-producing animals, and also in exploring key drivers for the incorporation of 'new' virulence and antimicrobial resistance genes into EAEC strains, thereby enhancing their spread and persistence in cases of human infection. These requirements are shown below.

7.3.1. Research needs

- PCR-based methods for detection and quantification of EAEC in food items should be further developed as this would assist the assessment of human exposure, e.g. in a foodborne outbreak. Similarly, a standardised and validated multiplex approach to the identification of the causal agents of diarrhoeal illnesses that involve multiple pathogens is required.
- Adaptation of such methods for animal samples should be considered.
- As *E. coli* strains possessing new combinations of virulence factors and AMR genes are emerging in foodborne outbreaks and infections, identification of drivers for incorporation of, e.g. *stx* genes into EAEC strains and the resultant fitness cost/benefits to strains is an important research requirement.
- To better understand the routes by which humans are expored to EAEC, methods for typing and comparative analysis of isolates collected from all potential reservoirs should be developed.
- The use of culture media containing customised combinations of relevant antimicrobials to enhance detection of multiple drug-resistant EAEC amongst mixed populations of organisms should be investigated and optimised.
- Further controlled studies are needed to fully quantify the survival characteristics of EAEC in wet and dry substrates under laboratory and natural conditions.
- Biofilm formation amongst EAEC strains is variable and involves several gene combinations. Since the ability to form biofilms is linked to the severity of human disease, more research is needed to elucidate this aspect.
- In order to gain a quantitative assessment of the occurrence of AMR in EAEC and the primary sources of resistance genes in EAEC isolates, including EAEC isolates from, e.g. UTIs, a more definitive statistically-based study of AMR in pathogenic *E. coli* serogroups principally associated with human infections, in comparison with those associated with foods and other possible sources, should be undertaken.

7.3.2. Surveillance needs

- Because of the increasing evidence of the involvement of EAEC in severe disease, surveillance of EAEC in human diarrhoeal infection should be improved.
- The evidence for animals and/or food being an important source of EAEC is sparse and is often based on the identification of genes such as *astA*, which are not specific for EAEC, or in studies performed in parts of the world where pollution by human faecal waste is common. To quantify the possible involvement in the EU MSs of EAEC strains in foods originating from such countries, a survey of imported non-EU foods should be considered.
- In view of the potential for EAEC to provide an enhanced platform for attachment of highly pathogenic *E. coli* strains to the human intestine, and because occurrence and prevalence data in food animals are sparse, testing for EAEC genes in animal intestinal or food samples already available from the harmonised monitoring of AMR in the EU would provide data for risk assessment.

- A standardised and validated gene-based protocol should be utilised to explore samples from the main livestock animal species (cattle, pigs, poultry) and the products derived from them for the presence of EAEC and zoonotic *E. coli*. Similarly, sampling protocols for vegetables, herbs and fruits should be developed and applied accordingly.
- Monitoring programmes or surveys should cover areas such as carrier status of humans, particularly food handlers, and animals; and occurrence in foods (including foods of non-animal origin) and the environment.
- In light of the apparently increasing involvement of EAEC in mixed pathogen outbreaks, surveillance of foods associated with such outbreaks for EAEC should be encouraged.
- To better understand the pathways EAEC strains take to cause infections, isolates should be collected from different potential reservoirs and comparatively analysed.
- PCR-based methods for detection and quantification of EAEC in food items should be made available, as this would assist the exposure assessment of persons at risk, e.g. in a foodborne outbreak.

8. Conclusions

8.1. General

Enteroaggregative *Escherichia coli* (EAEC) are a highly complex group of organisms belonging to a wide range of serogroups and serotypes. Such strains carry a range of virulence factors and often antimicrobial resistance (AMR) genes. They frequently acquire new virulence genes which may enhance their pathogenicity and make definitive categorisation based on the presence or absence of specific virulence determinants difficult.

EAEC are a major cause of acute diarrhoeal illness in infants in many low-income countries world-wide and also in travellers both to and returning from such countries. In higher-income countries EAEC are increasingly involved in food-related incidents and outbreaks, and in non-gastroenteric infections such as urinary tract infections (UTIs).

There is considerable uncertainty about the numbers of EAEC infections in EU Member States (MSs) each year.

A number of polymerase chain reaction (PCR)-based assays that have displayed a good performance characteristics are available and have been effectively used for the diagnosis of EAEC infections. A good candidate method for the detection of EAEC in foods has been developed by the EU Reference Laboratory (RL) verocytotoxin-producing *E. coli* (VTEC) and used in the EU RL VTEC Proficiency Testing schemes. When food samples are shown to be positive for EAEC, isolation of the organism(s) present should be undertaken to facilitate epidemiological studies

Current evidence indicates that in EU MSs EAEC are primarily non-zoonotic in origin and that transmission mainly occurs by person-to-person spread and by the contamination of foods by asymptomatic carriers.

8.2. Answers to Terms of Reference

8.2.1. Answers to Term of Reference 1

- Gatroenteric *Escherichia coli* strains are conventionally divided into six pathotypes based on their pathogenicity profiles. These are: enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC) (including *Shigella* spp.), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC).
- As some pathotypes can belong to more than one serotype, serotyping may not provide definitive identification of pathotypes.
- An individual *E. coli* strain possessing virulence genes typical of more than one pathotype may be isolated from cases of disease, thereby presenting difficulties in epidemiological investigation.

- EAEC are characterised by their ability to adhere to tissue culture cells in a distinct stacked-brick pattern which is mediated by aggregative adherence fimbriae (AAF), of which there are several known isoforms (I, II, III, IV and V).
- Pathotype-determining factors for EAEC include *aggR, aaiC, aggA, aafA, agg3A, agg4A, agg5A,* AAF/I-V.
- Expression of AAF is mediated by the plasmid-encoded transcriptional activator AggR; cell attachment is also mediated by the toxigenic invasion locus A (Tia).
- EAEC often produce an enteroaggregative heat-stable toxin (EAST1) encoded by the plasmid-borne *astA* genes as well as *Shigella* enterotoxin (ShET1) and Haemolysin E (HlyE).
- EAEC also possess genes that encode a number of serine protease autotransporters of *Enterobacteriaceae* (SPATE) that are implicated in immune evasion, mucosal damage, secretogenicity, and colonization.
- The acquisition of virulence traits present in other pathotypes by EAEC can result in the formation of new, highly virulent organisms.
- EAEC lineages have evolved independently *via* multiple genetic events. The EAEC pan-genome is considered open and is still evolving by gene acquisition and diversification.

8.2.2. Answers to Term of Reference 2

- EAEC have been associated with travellers' diarrhoea and with acute diarrhoeal illness among children in both low-income and high-income regions, with severe diarrhoeal infections in immunocompromised patients, and with UTI infections.
- The clinical manifestations of EAEC infection vary from individual to individual, depending upon the genetic composition of the host and of the strain. Infections with EAEC may be asymptomatic, with persons carrying such strains exhibiting no overt disease symptoms.
- A predominant feature of EAEC infection in low-income countries is the propensity to cause persistent diarrhoea for more than 2 weeks, making these bacteria a significant cause of mortality, particularly in children.
- Stx-producing EAEC such as the 2011 *E. coli* O104:H4 outbreak strain have been associated with a range of symptoms such as haemolytic uraemic syndrome (HUS) and haemorrhagic colitis (HC), and have resulted in deaths in infected individuals.
- The emergence of mixed EAEC/STEC pathotype *E. coli* is likely to be an ongoing low frequency event and the occurrence of outbreaks probably relates primarily to opportunities for growth and dissemination of the organism(s) in foodstuffs or infected carriers.
- Strains of the EAEC pathotype are considered to be adapted to the human host. There is no evidence for animals being a reservoir of EAEC in EU MSs. Putative carriage of EAEC by animals is often based on the reporting of genes such as *astA*, which are not specific for EAEC, in parts of the world where pollution by human faecal waste is common.
- Outbreaks of EAEC associated with foods are frequently suggestive of contamination by asymptomatic food handlers and to poor sanitation.
- Multiple pathogen outbreaks in which a range of pathogens as well as EAEC are implicated are being increasingly identified.
- Contamination of the environment, particularly watercourses, can occur in parts of the world where human sanitary systems are insufficient and EAEC is prominent in people. Environmental contamination may also be a pathway for EAEC occurence on produce.
- Prolonged survival of EAEC for at least several weeks in wet and dry substrates appears to be possible, but further controlled studies under laboratory and natural conditions are needed to fully quantify this.

- Biofilm formation amongst EAEC strains is variable and involves several gene combinations. The ability to form biofilms is linked to the severity of human disease and is likely to be involved in survival in the environment.

8.2.3. Answers to Term of Reference 3

- Testing of both food and faecal samples involves the detection of EAEC-associated traits in the matrix or in enrichment culture from such matrices by molecular methods, followed by isolation of the organism and confirmation of the presence of EAEC-associated genes or phenotypes.
- The most widely accepted diagnostic option for confirming EAEC isolates from a technical point of view remains the adhesion assay onto monolayers of cultured epithelial cells. This approach is cumbersome, expensive and requires experienced personnel, and is therefore confined to the reference laboratories.
- The widespread use of PCR combined with the increased availability of information on the virulence gene asset of EAEC has led to the development of a number of gene-based assays that have performed well. Such assays have been effectively used for the confirmation of EAEC infections.
- There is no consensus on a single PCR assay to be used in the routine testing of EAEC. The most recent approaches attempting to standardise EAEC identification revolve around the use of PCR amplification of specific markers from the plasmid and the chromosome.
- PCR-based methods can be used for the analyses of food for the presence of EAEC. In this respect the protocol developed by the EU RL VTEC for the detection of EAEC in food by Real Time PCR amplification of the *aggR* and *aaiC* genes and used in the EU RL VTEC Proficiency Testing schemes is presently considered a good candidate for the molecular detection of such organisms in food matrices by EU MSs.
- Whole genome sequencing (WGS) can provide information on the population structure of EAEC using a whole genome MLST (wgMLST) approach or the analysis of single nucleotide polymorphisms (SNPs). WGS can also determine the presence or absence of all the major putative EAEC virulence genes.
- WGS has not been used routinely for the identification of EAEC from either cases of human infection or from foods. Further studies are required to assess the potential of WGS for such purposes.

8.2.4. Answers to Term of Reference 4

- AMR is increasingly reported on a world-wide basis in isolates of EAEC in cases of sporadic diarrhoea in children and adults in both low-income and high-income countries, in returning travellers, and in outbreaks of infection.
- Antimicrobials such as ampicillin, co-trimoxazole and doxycycline have been used to treat EAEC infections. Fluoroquinolones and third-generation cephalosporins have been increasingly used, but the emergence of multiple antibiotic-resistant strains often coupled with resistance to quinolones and third-generation cephalosporins has compromised treatment.
- Food-borne outbreaks of EAEC in which the causative strains have exhibited AMR have been reported in recent years. The origin of the resistance genes in such strains has not been conclusively established.
- EAEC are more likely to exhibit resistance to antimicrobials than other pathogenic *E. coli*, such as STEC. Such differences may be strain-related or associated with differences in antimicrobial selective pressure.
- EAEC frequently exhibit resistance to one or more of the antimicrobials commonly used in human medicine. As such, the use of aforesaid antimicrobials will enhance their survival and spread in ecosystems where such agents are deployed.

8.2.5. Answers to Term of Reference 5

Research needs

- PCR-based methods for detection and quantification of EAEC in food items should be further developed, as this would assist the assessment of human exposure, e.g. in a foodborne outbreak. Similarly, a standardised and validated multiplex approach to the identification of the causal agents of diarrhoeal illnesses that involve multiple pathogens is required.
- Adaptation of such methods for animal samples should be considered.
- As *E. coli* strains possessing new combinations of virulence factors and AMR genes are emerging in foodborne outbreaks and infections, identification of drivers for incorporation of, e.g. *stx* genes into EAEC strains and the resultant fitness cost/benefits to strains is an important research requirement.
- To better understand the routes by which humans are exposed to EAEC, methods for typing and comparative analysis of isolates collected from all potential reservoirs should be developed.
- The use of culture media containing customised combinations of relevant antimicrobials to enhance detection of multiple drug-resistant EAEC amongst mixed populations of organisms should be investigated and optimised.
- Further controlled studies are needed to fully quantify the survival characteristics of EAEC in wet and dry substrates under laboratory and natural conditions.
- Biofilm formation amongst EAEC strains is variable and involves several gene combinations. Since the ability to form biofilms is linked to the severity of human disease, more research is needed to elucidate this aspect.
- In order to gain a quantitative assessment of the occurrence of AMR in EAEC and the primary sources of resistance genes in EAEC isolates, including EAEC isolates from, e.g. UTIs, a more definitive statistically-based study of AMR in pathogenic *E. coli* serogroups, principally associated with human infections in comparison to those associated with animals and other possible reservoirs, should be undertaken.

Surveillance needs

- Because of the increasing evidence of the involvement of EAEC in severe disease, surveillance of EAEC in human diarrhoeal infection should be improved.
- The evidence for animals and/or food being an important source of EAEC is sparse and is often based on the identification of genes such as *astA*, which are not specific for EAEC, or in studies performed in parts of the world where pollution by human faecal waste is common. To quantify the possible involvement in the EU MSs of EAEC strains originating from such countries, a survey of imported non-EU foods should be considered.
- In view of the potential for EAEC to provide an enhanced platform for attachment of highly pathogenic *E. coli* strains to the human intestine, and because occurrence and prevalence data in food animals are sparse, testing for EAEC genes in animal intestinal samples already available from the harmonised monitoring of AMR in the EU would provide data for risk assessment.
- Monitoring programmes or surveys should cover aspects such as carrier status of humans, particularly food handlers, and animals; and occurrence in foods (including foods of non-animal origin) and the environment.
- A standardised and validated gene-based protocol should be utilised to explore samples from the main livestock animal species (cattle, pigs, poultry) and the products derived from them for the presence of EAEC and zoonotic *E.coli*. Similarly, sampling protocols for vegetables, herbs and fruits should be developed and applied accordingly.

- In light of the apparently increasing involvement of EAEC in mixed pathogen outbreaks, surveillance of foods associated with such outbreaks for EAEC should be encouraged.
- To better understand the pathways EAEC strains take to cause infections, isolates should be collected from different potential reservoirs and comparatively analysed.
- PCR-based methods for detection and quantification of EAEC in food items should be made available, as this would assist the exposure assessment of persons at risk, e.g. in a foodborne outbreak.

9. Recommendations

- Implementation of a multiplex gastrointestinal (GI) PCR approach for the detection of multiple GI pathogens, including EAEC. Such assays should provide a rapid, and cost-effective multi-pathogen approach for the detection of bacteria, viruses and parasites commonly associated with GI infection.
- The protocol developed by the EU RL VTEC for the detection of EAEC in food by Real Time PCR amplification of the *aggR* and *aaiC* genes is considered a good candidate for the molecular detection of such organisms in food matrices, and should be ratified for use by EU MSs.
- Following detection of EAEC genes in food matrices by molecular methods, efforts should be made to isolate the organism(s) in order to facilitate epidemiological investigations.
- In cases of food- and water-borne outbreaks of diarrhoeal disease with unknown etiology, the inclusion of EAEC in the screening of infected individuals and foods should be encouraged.
- The use of WGS for the identification of EAEC in food matrices and food-borne outbreaks should be further explored.

References

Abe CM, Salvador FA, Falsetti IN, Vieira MAM, Blanco J, Blanco JE, Blanco M, Machado AMO, Elias WP, Hernandes RT and Gomes TAT, 2008. Uropathogenic *Escherichia coli* (UPEC) strains may carry virulence properties of diarrhoeagenic *E. coli*. FEMS Immunology and Medical Microbiology, 52, 397-406.

Adachi JA, Jiang ZD, Mathewson JJ, Verenkar MP, Thompson S, Martinez-Sandoval F, Steffen R, Ericsson CD and DuPont HL, 2001. Enteroaggregative *Escherichia coli* as a major etiologic agent in traveler's diarrhea in 3 regions of the world. Clinical Infectious Diseases, 32, 1706-1709.

Akiyama Y, Saito E, Futai H, Ogita K, Sakae H, Fukunaga M, Tsuji H, Chikahira M and Mimura M, 2015. Comprehensive study of pathogenic genes distributed in *Escherichia coli* isolated from cattle. Food Hygiene and Safety Science, 56, 118-122.

Akter S, Islam M, Afreen KS, Azmuda N, Khan SI and Birkeland NK, 2013. Prevalence and distribution of different diarrhoeagenic *Escherichia coli* virulotypes in major water bodies in Bangladesh. Epidemiology and Infection, 141, 2516-2525.

Al Safadi R, Abu-Ali GS, Sloup RE, Rudrik JT, Waters CM, Eaton KA and Manning SD, 2012. Correlation between *in vivo* biofilm formation and virulence gene expression in *Escherichia coli* O104:H4. PLoS One, 7, e41628.

Alikhani MY, Hashemi SH, Aslani MM and Farajnia S, 2013. Prevalence and antibiotic resistance patterns of diarrheagenic *Escherichia coli* isolated from adolescents and adults in Hamedan, Western Iran. Iranian Journal of Microbiology, 5, 42-47.

Alves JR, Pereira ACM, Souza MC, Costa SB, Pinto AS, Mattos-Guaraldi AL, Hirata-Junior R, Rosa ACP and Asad LMBO, 2010. Iron-limited condition modulates biofilm formation and interaction with human epithelial cells of enteroaggregative *Escherichia coli* (EAEC). Journal of Applied Microbiology, 108, 246-255.

Alvim Liberatore AM, Tomita SK, Midolli Vieira MA, Toti CJr, Heckmaier J and Tardelli Gomes TA, 2007. Expression of aggregative adherence to HeLa cells by *Escherichia coli* strains isolated from sick horses. Brazilian Journal of Microbiology, 38, 9-13.

Amaya E, Reyes D, Vilchez S, Paniagua M, Mollby R, Nord CE and Weintraub A, 2011. Antibiotic resistance patterns of intestinal *Escherichia coli* isolates from Nicaraguan children. Journal of Medical Microbiology, 60, 216-222.

Andrade FB, Gomes TA and Elias WP, 2014. A sensitive and specific molecular tool for detection of both typical and atypical enteroaggregative *Escherichia coli*. Journal of Microbiological Methods, 106, 16-18.

Andrzejewska B and Sobieszczanska BM, 2013. Influence of Lactobacillus casei on biofilm formation by enteroaggregative *Escherichia coli* strains isolated from irritable bowel syndrome (IBS). Medycyna doswiadczalna i mikrobiologia, 65, 11-17.

Aranda KR, Fabbricotti SH, Fagundes-Neto U and Scaletsky IC, 2007. Single multiplex assay to identify simultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing *Escherichia coli* strains in Brazilian children. FEMS Microbiology Letters, 267, 145-150.

Araujo ANd and Giugliano LG, 1999. Human milk fractions inhibit the adherence of diffusely adherent *Escherichia coli* (DAEC) and enteroaggregative *E. coli* (EAEC) to HeLa cells. FEMS Microbiology Letters, 184, 91-94.

Araujo JM, Tabarelli GF, Aranda KRS, Fabbricotti SH, Fagundes-Neto U, Mendes CMF and Scaletsky ICA, 2007. Typical enteroaggregative and atypical enteropathogenic types of *Escherichia coli* are the most prevalent diarrhea-associated pathotypes among Brazilian children. Journal of Clinical Microbiology, 45, 3396-3399.

Arvand M, Bettge-Weller G, Fruth A, Uphoff H and Pfeifer Y, 2015. Extended-spectrum beta-lactamase-producing Shiga toxin gene (*stx1*)-positive *Escherichia coli* O91:H14 carrying $bla_{CTX\text{-}M\text{-}1}$ on an IncI1-ST31 plasmid isolated from a human patient in Germany. International Journal of Medical Microbiology, 305, 404-407.

Aslani MM, Alikhani MY, Zavari A, Yousefi R and Zamani AR, 2011. Characterization of enteroaggregative *Escherichia coli* (EAEC) clinical isolates and their antibiotic resistance pattern. International Journal of Infectious Diseases, 15, E136-E139.

Aurass P, Prager R and Flieger A, 2011. EHEC/EAEC O104:H4 strain linked with the 2011 German outbreak of haemolytic uremic syndrome enters into the viable but non-culturable state in response to various stresses and resuscitates upon stress relief. Environmental Microbiology, 13, 3139-3148.

Auvray F, Dilasser F, Bibbal D, Kérourédan M, Oswald E and Brugère H, 2012. French cattle is not a reservoir of the highly virulent enteroaggregative Shiga toxin-producing *Escherichia coli* of serotype O104:H4. Veterinary Microbiology, 158, 443-445.

Bangar R and Mamatha B, 2008. Identification of enteroaggregative *Escherichia coli* in infants with acute diarrhea based on biofilm production in Manipal, south India. Indian Journal of Medical Sciences, 62, 8-12.

Baranzoni GM, Fratamico PM, Rubio F, Glaze T, Bagi LK and Albonetti S, 2014. Detection and isolation of Shiga toxin-producing *Escherichia coli* (STEC) O104 from sprouts. International Journal of Food Microbiology, 173, 99-104.

Baudry B, Savarino SJ, Vial P, Kaper JB and Levine MM, 1990. A sensitive and specific DNA probe to identify enteroaggregative *Escherichia coli*, a recently discovered diarrheal pathogen. The Journal of Infectious Diseases, 161, 1249-1251.

Benevides-Matos N, Pieri FA, Penatti M and Orlandi PP, 2015. Adherence and virulence genes of *Escherichia coli* from children diarrhoea in the Brazilian Amazon. Brazilian Journal of Microbiology, 46, 131-137.

Berger CN, Shaw RK, Ruiz-Perez F, Nataro JP, Henderson IR, Pallen MJ and Frankel G, 2009. Interaction of enteroaggregative *Escherichia coli* with salad leaves. Environmental Microbiology Reports, 1, 234-239.

Berry AA, Yang Y, Pakharukova N, Garnett JA, Lee W-c, Cota E, Marchant J, Roy S, Tuittila M, Liu B, Inman KG, Ruiz-Perez F, Mandomando I, Nataro JP, Zavialov AV and Matthews S, 2014. Structural insight into host recognition by aggregative adherence fimbriae of Enteroaggregative *Escherichia coli*. PLoS Pathogens, 10, e1004404.

Beutin L, Hammerl JA, Reetz J and Strauch E, 2013. Shiga toxin-producing *Escherichia coli* strains from cattle as a source of the Stx2a bacteriophages present in enteroaggregative *Escherichia coli* 0104:H4 strains. International Journal of Medical Microbiology, 303, 595-602.

Bhan MK, Bhandari N, Sazawal S, Clemens J, Raj P, Levine MM and Kaper JB, 1989. Descriptive epidemiology of persistent diarrhea among young-children in rural northern India. Bulletin of the World Health Organization, 67, 281-288.

Bibbal D, Kerouredan M, Loukiadis E, Scheutz F, Oswald E and Brugere H, 2014. Slaughterhouse effluent discharges into rivers not responsible for environmental occurrence of enteroaggregative *Escherichia coli*. Veterinary Microbiology, 168, 451-454.

Bielaszewska M, Mellmann A, Zhang WL, Kock R, Fruth A, Bauwens A, Peters G and Karch H, 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. The Lancet. Infectious Diseases, 11, 671-676.

Binnicker MJ, 2015. Multiplex molecular panels for the diagnosis of gastrointestinal infection: performance, result interpretation and cost-effectiveness. Journal of Clinical Microbiology.

Boisen N, Melton-Celsa AR, Scheutz F, O'Brien AD and Nataro JP, 2015. Shiga toxin 2a and Enteroaggregative *Escherichia coli* - a deadly combination. Gut Microbes, 6, 272-278.

Boisen N, Ruiz-Perez F, Scheutz F, Krogfelt KA and Nataro JP, 2009. Short report: high prevalence of serine protease autotransporter cytotoxins among strains of enteroaggregative *Escherichia coli*. American Journal of Tropical Medicine and Hygiene, 80, 294-301.

Boisen N, Scheutz F, Rasko DA, Redman JC, Persson S, Simon J, Kotloff KL, Levine MM, Sow S, Tamboura B, Toure A, Malle D, Panchalingam S, Krogfelt KA and Nataro JP, 2012. Genomic characterization of enteroaggregative *Escherichia coli* from children in Mali. Journal of Infectious Diseases, 205, 431-444.

Bolick DT, Roche JK, Hontecillas R, Bassaganya-Riera J, Nataro JP and Guerrant RL, 2013. Enteroaggregative *Escherichia coli* strain in a novel weaned mouse model: exacerbation by malnutrition, biofilm as a virulence factor and treatment by nitazoxanide. Journal of Medical Microbiology, 62, 896-905.

Boll EJ, Struve C, Boisen N, Olesen B, Stahlhut SG and Krogfelt KA, 2013. Role of enteroaggregative *Escherichia coli* virulence factors in uropathogenesis. Infection and Immunity, 81, 1164-1171.

Boudailliez B, Berquin P, Mariani-Kurkdjian P, Ilef D D, Cuvelier B, Capek I, Tribout B, Bingen E and Piussan C, 1997. Possible person-to-person transmission of *Escherichia coli* O111 associated hemolytic uremic syndrome. Pediatric Nephrology, 36-39.

Bouzari S, Jafari A, Azizi A, Oloomi M and Nataro JP, 2001. Short report: characterization of enteroaggregative *Escherichia coli* isolates from Iranian children. American Journal of Tropical Medicine and Hygiene, 65, 13-14.

Brandal LT, Lindstedt BA, Aas L, Stavnes TL, Lassen J and Kapperud G, 2007. Octaplex PCR and fluorescence-based capillary electrophoresis for identification of human diarrheagenic *Escherichia coli* and *Shigella* spp. Journal of Microbiological Methods, 68, 331-341.

Breitwieser F, 1999. Studies about the pathogenicity of haemolytic and non-haemolytic *Escherichia coli* out of samples from dogs and cats which had fallen sick with enteritis or perished. Tierarztliche Praxis Ausgabe Kleintiere Heimtiere, 27, 381-385.

Brenner DJ, Fanning GR, Steigerwalt AG, Orskov I and Orskov F, 1972. Poly-nucleotide sequence relatedness among 3 groups of pathogenic *Escherichia coli* strains. Infection and Immunity, 6, 308-315.

Brenner DJ, Steigerwalt AG, Wathen HG, Gross RJ and Rowe B, 1982. Confirmation of aerogenic strains of *Shigella boydii*-13 and further study of *Shigella* serotypes by DNA relatedness. Journal of Clinical Microbiology, 16, 432-436.

Brzuszkiewicz E, Thurmer A, Schuldes J, Leimbach A, Liesegang H, Meyer FD, Boelter J, Petersen H, Gottschalk G and Daniel R, 2011. Genome sequence analyses of two isolates from the recent *Escherichia coli* outbreak in Germany reveal the emergence of a new pathotype: Entero-Aggregative-Haemorrhagic *Escherichia coli* (EAHEC). Archives of Microbiology, 193, 883-891.

Buchholz U, Bernard H, Werber D, Bohmer MM, Remschmidt C, Wilking H, Delere Y, an der Heiden M, Adlhoch C, Dreesman J, Ehlers J, Ethelberg S, Faber M, Frank C, Fricke G, Greiner M, Hohle M, Ivarsson S, Jark U, Kirchner M, Koch J, Krause G, Luber P, Rosner B, Stark K and Kuhne M, 2011. German outbreak of *Escherichia coli* O104:H4 associated with sprouts. New England Journal of Medicine, 365, 1763-1770.

Buvens G, De Gheldre Y, Dediste A, de Moreau AI, Mascart G, Simon A, Allemeersch D, Scheutz F, Lauwers S and Pierard D, 2012. Incidence and Virulence Determinants of Verocytotoxin-Producing *Escherichia coli* Infections in the Brussels-Capital Region, Belgium, in 2008-2010. Journal of Clinical Microbiology, 50, 1336-1345.

Carbonari C, Miliwebsky E, Deza N, Zolezzi G, Manfredi E, D'Astek BA, Baschkier A, Chinen I and Rivas M, 2015. Novel EAEC/STEC hybrid O59:NM[H19] strains isolated from human infections in Argentina. Abstract: VTEC2015 Boston (USA), 14 Sept. 2015, p. 38. Available online: http://www.vtec2015.org/wp-content/uploads/2015/09/E-Abstracts-Oral-Poster-21.pdf

Cassar CA, Ottaway M, Paiba GA, Futter R, Newbould S and Woodward MJ, 2004. Absence of enteroaggregative *Escherichia coli* in farmed animals in Great Britain. Veterinary Record, 154, 237-239.

Castro-Rosas J, Cerna-Cortes JF, Mendez-Reyes E, Lopez-Hernandez D, Gomez-Aldapa CA and Estrada-Garcia T, 2012. Presence of faecal coliforms, *Escherichia coli* and diarrheagenic *E. coli* pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. International Journal of Food Microbiology, 156, 176-180.

CDC (Centers for Disease Control and Prevention), 2011. CDC Estimates of Foodborne Illness in the United States. Available online: http://www.cdc.gov/foodborneburden/PDFs/FACTSHEET_A_FINDINGS_updated4-13.pdf

Cennimo DJ, Koo H, Mohamed JA, Huang DB and Chiang T, 2007. Enteroaggregative *Escherichia coli*: A review of trends, diagnosis, and treatment. Infections in Medicine, 24, 100-110.

Cerna JF, Nataro JP and Estrada-Garcia T, 2003. Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative *Escherichia coli* strains. Journal of Clinical Microbiology, 41, 2138-2140.

Chattaway MA, Harris R, Jenkins C, Tam C, Coia JE, Gray J, Iturriza-Gomara M and Wain J, 2013. Investigating the link between the presence of enteroaggregative *Escherichia coli* and infectious intestinal disease in the United Kingdom, 1993 to 1996 and 2008 to 2009. Eurosurveillance, 18, 20-26.

Chattaway MA, Jenkins C, Ciesielczuk H, Day M, DoNascimento V, Day M, Rodriguez I, van Essen-Zandbergen A, Schink AK, Wu GH, Threlfall J, Woodward MJ, Coldham N, Kadlec K, Schwarz S, Dierikx C, Guerra B, Helmuth R, Mevius D, Woodford N and Wain J, 2014a. Evidence of evolving extraintestinal enteroaggregative *Escherichia coli* ST38 clone. Emerging Infectious Diseases, 20, 1935-1937.

Chattaway MA, Jenkins C, Rajendram D, Cravioto A, Talukder KA, Dallman T, Underwood A, Platt S, Okeke IN and Wain J, 2014b. Enteroaggregative *Escherichia coli* have evolved independently as distinct complexes within the *E. coli* population with varying ability to cause disease. PLoS One, 9.

Chaudhuri RR, Sebaihia M, Hobman JL, Webber MA, Leyton DL, Goldberg MD, Cunningham AF, Scott-Tucker A, Ferguson PR, Thomas CM, Frankel G, Tang CM, Dudley EG, Roberts IS, Rasko DA, Pallen

MJ, Parkhill J, Nataro JP, Thomson NR and Henderson IR, 2010. Complete genome sequence and comparative metabolic profiling of the prototypical enteroaggregative *Escherichia coli* strain 042. PLoS One, 5, e8801.

Chen Y, Chen X, Zheng S, Yu F, Kong H, Yang Q, Cui D, Chen N, Lou B, Li X, Tian L, Yang X, Xie G, Dong Y, Qin Z, Han D, Wang Y, Zhang W, Tang YW and Li L, 2014. Serotypes, genotypes and antimicrobial resistance patterns of human diarrhoeagenic *Escherichia coli* isolates circulating in southeastern China. Clinical Microbiology and Infection, 20, 52-58.

Clements A, Young JC, Constantinou N and Frankel G, 2012. Infection strategies of enteric pathogenic *Escherichia coli*. Gut Microbes, 3, 71-87.

Cordeiro F, da Silva Gomes Pereira D, Rocha M, Asensi MD, Elias WP and Campos LC, 2008. Evaluation of a multiplex PCR for identification of enteroaggregative *Escherichia coli*. Journal of Clinical Microbiology, 46, 828-829.

Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M and Finlay BB, 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. Clinical Microbiology Reviews, 26, 822-880.

Czeczulin JR, Whittam TS, Henderson IR, Navarro-Garcia F and Nataro JP, 1999. Phylogenetic analysis of enteroaggregative and diffusely adherent *Escherichia coli*. Infection and Immunity, 67, 2692-2699.

Dacko J, Weiner M and Osek J, 2004. Classifying *Escherichia coli* strains isolated from piglets diagnosed with diarrhea through amplifying virulence markers with PCR. Medycyna Weterynaryjna, 60, 952-957.

Dallman T, Smith GP, O'Brien B, Chattaway MA, Finlay D, Grant KA and Jenkins C, 2012. Characterization of a verocytotoxin-producing Enteroaggregative *Escherichia coli* serogroup O111:H21 strain associated with a household outbreak in Northern Ireland. Journal of Clinical Microbiology, 50, 4116-4119.

Dallman TJ, Chattaway MA, Cowley LA, Doumith M, Tewolde R, Wooldridge DJ, Underwood A, Ready D, Wain J, Foster K, Grant KA and Jenkins C, 2014. An investigation of the diversity of strains of enteroaggregative *Escherichia coli* isolated from cases associated with a large multi-pathogen foodborne outbreak in the UK. PLoS One, 9.

Darfeuille-Michaud A, 2002. Adherent-invasive *Escherichia coli*: a putative new *E. coli* pathotype associated with Crohn's disease. International Journal of Medical Microbiology, 292, 185-193.

Dobrowsky PH, van Deventer A, De Kwaadsteniet M, Ndlovu T, Khan S, Cloete TE and Khan W, 2014. Prevalence of virulence genes associated with pathogenic *Escherichia coli* strains isolated from domestically harvested rainwater during low- and high-rainfall periods. Applied and Environmental Microbiology, 80, 1633-1638.

dos Santos LF, Goncalves EM, Vaz TMI and Guth BEC, 2007. Distinct pathotypes of O113 *Escherichia coli* strains isolated from humans and animals in Brazil. Journal of Clinical Microbiology, 45, 2028-2030.

Dudley EG, Abe C, Ghigo JM, Latour-Lambert P, Hormazabal JC and Nataro JP, 2006a. An IncI1 plasmid contributes to the adherence of the atypical enteroaggregative *Escherichia coli* strain C1096 to cultured cells and abiotic surfaces. Infection and Immunity, 74, 2102-2114.

Dudley EG, Thomson NR, Parkhill J, Morin NP and Nataro JP, 2006b. Proteomic and microarray characterization of the AggR regulon identifies a pheU pathogenicity island in enteroaggregative *Escherichia coli*. Molecular Microbiology, 61, 1267-1282.

Duffy LL, O'Callaghan D, McAuley CM, Fegan N and Craven HM, 2009. Virulence properties of *Escherichia coli* isolated from Australian dairy powder factory environments. International Dairy Journal, 19, 178-179.

Dutta PR, Cappello R, Navarro-Garcia F and Nataro JP, 2002. Functional comparison of serine protease autotransporters of *Enterobacteriaceae*. Infection and Immunity, 70, 7105-7113.

Dutta S, Pal S, Chakrabarti S, Dutta P and Manna B, 1999. Use of PCR to identify enteroaggregative *Escherichia coli* as an important cause of acute diarrhoea among children living in Calcutta, India. Journal of Medical Microbiology, 48, 1011-1016.

EFSA (European Food Safety Authority), 2011a. Shiga toxin-producing *E. coli* (STEC) O104:H4 2011 outbreaks in Europe: taking stock. EFSA Journal 2011;9(10):2390, 22 pp. doi:10.2903/j.efsa.2011.2390

EFSA (European Food Safety Authority), 2011b. Tracing seeds, in particular fenugreek (*Trigonella foenum-graecum*) seeds, in relation to the Shiga toxin-producing *E. coli* (STEC) O104:H4 2011 outbreaks in Germany and France.

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2013. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011. EFSA Journal 2013;11(4):3129, 250 pp. doi:10.2903/j.efsa.2013.3129.

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2013. Scientific Opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. EFSA Journal 2013;11(4):3138, 106 pp. doi:10.2903/j.efsa.2013.3138

Ennis C, McDowell D and Bolton DJ, 2012. The prevalence, distribution and characterization of Shiga toxin-producing *Escherichia coli* (STEC) serotypes and virulotypes from a cluster of bovine farms. Journal of Applied Microbiology, 113, 1238-1248.

Estrada-Garcia T and Navarro-Garcia F, 2012. Enteroaggregative *Escherichia coli* pathotype: a genetically heterogeneous emerging foodborne enteropathogen. FEMS Immunology and Medical Microbiology, 66, 281-298.

Evans J, Wilson A, Willshaw GA, Cheasty T, Tompkins DS, Wheeler JG and Smith HR, 2002. Vero cytotoxin-producing *Escherichia coli* in a study of infectious intestinal disease in England. Clinical Microbiology and Infection, 8, 183-186.

Fang GD, Lima AAM, Martins CV, Nataro JP and Guerrant RL, 1995. Etiology and epidemiology of persistent diarrhea in northeastern Brazil - a hospital-based, prospective, case-control study. Journal of Pediatric Gastroenterology and Nutrition, 21, 137-144.

Forster M, Messler S, Pfeffer K and Sievert K, 2009. Synanthropic flies as potential transmitters of pathogens to animals and humans. Mitteilungen der Deutschen Gesellschaft fur allgemeine und angewandte Entomologie, 17, 327-329.

Frank C, Werber D, Cramer JP, Askar M, Faber M, an der Heiden M, Bernard H, Fruth A, Prager R, Spode A, Wadl M, Zoufaly A, Jordan S, Kemper MJ, Follin P, Muller L, King LA, Rosner B, Buchholz U, Stark K, Krause G and Team HUSI, 2011. Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. The New England Journal of Medicine, 365, 1771-1780.

Franz E, Delaquis P, Morabito S, Beutin L, Gobius K, Rasko DA, Bono J, French N, Osek J, Lindstedt BA, Muniesa M, Manning S, LeJeune J, Callaway T, Beatson S, Eppinger M, Dallman T, Forbes KJ, Aarts H, Pearl DL, Gannon VP, Laing CR and Strachan NJ, 2014. Exploiting the explosion of information associated with whole genome sequencing to tackle Shiga toxin-producing *Escherichia coli* (STEC) in global food production systems. International Journal of Food Microbiology, 187, 57-72.

Fruth A, Prager R, Tietze E, Rabsch W and Flieger A, 2015. Molecular epidemiological view on Shiga toxin-producing *Escherichia coli* causing human disease in Germany: diversity, prevalence, and outbreaks. International Journal of Medical Microbiology, 305, 697-704.

FSA (Food Standards Agency), 2000. A report of the study of infectious intestinal disease in England. London: The Stationery Office. Available online: http://www.esds.ac.uk/doc/4092%5Cmrdoc%5Cpdf%5C4092userguide6.pdf

Fujioka M, Otomo Y and Ahsan CR, 2013. A novel single-step multiplex polymerase chain reaction assay for the detection of diarrheagenic *Escherichia coli*. Journal of Microbiological Methods, 92, 289-292.

Fujiyama R, Nishi J, Imuta N, Tokuda K, Manago K and Kawano Y, 2008. The shf gene of a *Shigella flexneri* homologue on the virulent plasmid pAA2 of enteroaggregative *Escherichia coli* 042 is required for firm biofilm formation. Current Microbiology, 56, 474-480.

Gascon J, Vargas M, Quinto L, Corachan M, de Anta MTJ and Vila J, 1998. Enteroaggregative *Escherichia coli* strains as a cause of traveler's diarrhea: a case-control study. Journal of Infectious Diseases, 177, 1409-1412.

Gilligan PH, 1999. *Escherichia coli*. EAEC, EHEC, EIEC, ETEC. Clinics in Laboratory Medicine, 19, 505-521.

Gomez-Aldapa CA, Rangel-Vargas E, Bautista-De Leon H, Estela Vazquez-Barrios M, Gordillo-Martinez AJ and Castro-Rosas J, 2013. Behavior of enteroaggregative *Escherichia coli*, non-O157-shiga toxin-producing *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli* and enterotoxigenic *E. coli* strains on mung bean seeds and sprout. International Journal of Food Microbiology, 166, 364-368.

Gomez-Duarte OG, Bai J and Newell E, 2009. Detection of *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae,* and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. Diagnostic Microbiology and Infectious Disease, 63, 1-9.

Grande L, Michelacci V, Tozzoli R, Ranieri P, Maugliani A, Caprioli A and Morabito S, 2014. Whole genome sequence comparison of *vtx2*-converting phages from enteroaggregative haemorrhagic *Escherichia coli* strains. BMC Genomics, 15, 574, doi:510.1186/1471-2164-1115-1574

Greenberg DE, Jiang ZD, Steffen R, Verenker MP and DuPont HL, 2002. Markers of inflammation in bacterial diarrhea among travelers, with a focus on enteroaggregative *Escherichia coli* pathogenicity. Journal of Infectious Diseases, 185, 944-949.

Guiral E, Mendez-Arancibia E, Soto SM, Salvador P, Fabrega A, Gascon J and Vila J, 2011. CTX-M-15-producing enteroaggregative *Escherichia coli* as cause of travelers' diarrhea. Emerging Infectious Diseases, 17, 1950-1953.

Gunzburg ST, Chang BJ, Elliott SJ, Burke V and Gracey M, 1993. Diffuse and enteroaggregative patterns of adherence of enteric *Escherichia coli* isolated from aboriginal children from the Kimberley region of Western Australia. Journal of Infectious Diseases, 167, 755-758.

Gyles CL, 1994. *Escherichia coli* in domestic animals and humans. CAB International, Wallingford, UK, 666 pp.

Haider K, Faruque SM, Albert MJ, Nahar S, Neogi PK and Hossain A, 1992. Comparison of a modified adherence assay with existing assay methods for identification of enteroaggregative *Escherichia coli*. Journal of Clinical Microbiology, 30, 1614-1616.

Harada T, Hiroi M, Kawamori F, Furusawa A, Ohata K, Sugiyama K and Masuda T, 2007. A food poisoning diarrhea outbreak caused by enteroaggregative *Escherichia coli* serogroup O126 : H27 in Shizuoka, Japan. Japanese Journal of Infectious Diseases, 60, 154-155.

Harrington SM, Dudley EG and Nataro JP, 2006. Pathogenesis of enteroaggregative *Escherichia coli* infection. FEMS Microbiology Letters, 254, 12-18.

Hebbelstrup Jensen B, Olsen KEP, Struve C, Krogfelt KA and Petersen AM, 2014. Epidemiology and clinical manifestations of enteroaggregative *Escherichia coli*. Clinical Microbiology Reviews, 27, 614-630.

Hedberg CW, Savarino SJ, Besser JM, Paulus CJ, Thelen VM, Myers LJ, Cameron DN, Barrett TJ, Kaper JB, Osterholm MT and Investigation team, 1997. An outbreak of foodborne illness caused by *Escherichia coli* O39:NM, an agent not fitting into the existing scheme for classifying diarrheogenic *E. coli*. Journal of Infectious Diseases, 176, 1625-1628.

Herzog K, Engeler Dusel J, Hugentobler M, Beutin L, Sagesser G, Stephan R, Hachler H and Nuesch-Inderbinen M, 2014. Diarrheagenic enteroaggregative *Escherichia coli* causing urinary tract infection and bacteremia leading to sepsis. Infection, 42, 441-444.

Huang DB and Dupont HL, 2004. Enteroaggregative *Escherichia coli*: an emerging pathogen in children. Seminars in Pediatric Infectious Diseases, 15, 266-271.

Huang DB, Mohamed JA, Nataro JP, DuPont HL, Jiang ZD and Okhuysen PC, 2007. Virulence characteristics and the molecular epidemiology of enteroaggregative *Escherichia coli* isolates from travellers to developing countries. Journal of Medical Microbiology, 56, 1386-1392.

Huang DB, Nataro JP, DuPont HL, Kamat PP, Mhatre AD, Okhuysen PC and Chiang T, 2006. Enteroaggregative *Escherichia coli* is a cause of acute diarrheal illness: A meta-analysis. Clinical Infectious Diseases, 43, 556-563.

Huang DB, Okhuysen PC, Jiang ZD and DuPont HL, 2004. Enteroaggregative *Escherichia coli*: an emerging enteric pathogen. American Journal of Gastroenterology, 99, 383-389.

Huang S-W, Hsu B-M, Su Y-J, Ji D-D, Lin W-C, Chen J-L, Shih F-C, Kao P-M and Chiu Y-C, 2011. Occurrence of diarrheagenic *Escherichia coli* genes in raw water of water treatment plants. Environmental Science and Pollution Research International, 19, 2776-2783.

Infante RM, Ericsson CD, Jiang ZD, Ke S, Steffen R, Riopel L, Sack DA and DuPont HL, 2004. Enteroaggregative *Escherichia coli* diarrhea in travelers: response to rifaximin therapy. Clinical Gastroenterology and Hepatology, 2, 135-138.

Itoh Y, Nagano I, Kunishima M and Ezaki T, 1997. Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable:H10 associated with a massive outbreak of gastrointestinal illness. Journal of Clinical Microbiology, 35, 2546-2550.

Iyoda S, Tamura K, Itoh K, Izumiya H, Ueno N, Nagata R, Togo M, Terajima J and Watanabe H, 2000. Inducible stx2 phages are lysogenized in the enteroaggregative and other phenotypic *Escherichia coli* O86:HNM isolated from patients. FEMS Microbiology Letters, 191, 7-10.

Jenkins C, Chart H, Willshaw GA, Cheasty T and Smith HR, 2006a. Genotyping of enteroaggregative *Escherichia coli* and identification of target genes for the detection of both typical and atypical strains. Diagnostic Microbiology and Infectious Disease, 55, 13-19.

Jenkins C, Tembo M, Chart H, Cheasty T, Willshaw GA, Phillips AD, Tompkins D and Smith H, 2006b. Detection of enteroaggregative *Escherichia coli* in faecal samples from patients in the community with diarrhoea. Journal of Medical Microbiology, 55, 1493-1497.

Jenkins C, van Ijperen C, Dudley EG, Chart H, Willshaw GA, Cheasty T, Smith HR and Nataro JP, 2005. Use of a microarray to assess the distribution of plasmid and chromosomal virulence genes in strains of enteroaggregative *Escherichia coli*. FEMS Microbiology Letters, 253, 119-124.

Jiang ZD, Okhuysen PC, Guo DC, He RM, King TM, DuPont HL and Milewicz DM, 2003. Genetic susceptibility to enteroaggregative *Escherichia coli* diarrhea: Polymorphism in the interleukin-8 promotor region. Journal of Infectious Diseases, 188, 506-511.

Kagambega A, Barro N, Traore AS, Siitonen A and Haukka K, 2012a. Characterization of *Salmonella enterica* and detection of the virulence genes specific to diarrheagenic *Escherichia coli* from poultry carcasses in Ouagadougou, Burkina Faso. Foodborne Pathogens and Disease, 9, 589-593.

Kagambega A, Martikainen O, Lienemann T, Siitonen A, Traore AS, Barro N and Haukka K, 2012b. Diarrheagenic *Escherichia coli* detected by 16-plex PCR in raw meat and beef intestines sold at local markets in Ouagadougou, Burkina Faso. International Journal of Food Microbiology, 153, 154-158.

Kagambega A, Martikainen O, Siitonen A, Traore AS, Barro N and Haukka K, 2012c. Prevalence of diarrheagenic *Escherichia coli* virulence genes in the feces of slaughtered cattle, chickens, and pigs in Burkina Faso. MicrobiologyOpen, 1, 276-284.

Kahali S, Sarkar B, Rajendran K, Khanam J, Yamasaki S, Nandy RK, Bhattacharya SK and Ramamurthy T, 2004. Virulence characteristics and molecular epidemiology of enteroaggregative *Escherichia coli* isolates from hospitalized diarrheal patients in Kolkata, India. Journal of Clinical Microbiology, 42, 4111-4120.

Kang G, Pulimood AB, Mathan MM and Mathan VI, 2001. Enteroaggregative *Escherichia coli* infection in a rabbit model. Pathology, 33, 341-346.

Kaper JB, Nataro JP and Mobley HLT, 2004. Pathogenic *Escherichia coli*. Nature Reviews Microbiology, 2, 123-140.

Karch H, Denamur E, Dobrindt U, Finlay BB, Hengge R, Johannes L, Ron EZ, Tonjum T, Sansonetti PJ and Vicente M, 2012. The enemy within us: lessons from the 2011 European *Escherichia coli* O104:H4 outbreak. EMBO Molecular Medicine, 4, 841-848.

Knoebl T, Saidenberg ABS, Moreno AM, Gomes TAT, Vieira MAM, Leite DS, Blanco JE and Ferreira AJP, 2011. Serogroups and virulence genes of *Escherichia coli* isolated from psittacine birds. Pesquisa Veterinaria Brasileira, 31, 916-921.

Knutton S, Shaw R, Phillips AD, Smith HR, Willshaw GA, Watson P and Price E, 2001. Phenotypic and genetic analysis of diarrhea-associated *Escherichia coli* isolated from children in the United Kingdom. Journal of Pediatric Gastroenterology and Nutrition, 33, 32-40.

Kohler CD and Dobrindt U, 2011. What defines extraintestinal pathogenic *Escherichia coli*? International Journal of Medical Microbiology, 301, 642-647.

Kong HS, Hong XP and Li XF, 2015. Current perspectivesin pathogenesis and antimicrobial resistance of enteroaggregative *Escherichia coli*. Microbial Pathogenesis, 85, 44-49.

Koo H-J, Kwak H-S, Yoon S-H and Woo G-J, 2012. Phylogenetic group distribution and prevalence of virulence genes in *Escherichia coli* isolates from food samples in South Korea. World Journal of Microbiology & Biotechnology, 28, 1813-1816.

Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque ASG, Zaidi AKM, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM and Levine MM, 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet, 382, 209-222.

Kunsmann L, Rüter C, Bauwens A, Greune L, Glüder M, Kemper B, Fruth A, Wai SN, He X, Lloubes R, Schmidt MA, Dobrindt U, Mellmann A, Karch H and Bielaszewska M, 2015. Virulence from vesicles: Novel mechanisms of host cell injury by *Escherichia coli* O104: H4 outbreak strain. Scientific Reports, 5, 13252.

Kuroda K, Suzuki R, Ihara K, Miyagi H, Watanabe H, Sato K, Hang'ombe BM, Mubita C, Isogai N, Mulenga E, Moonga L, Isogai H, Fukuda T, Yoneyama H and Isogai E, 2013. Detection of virulence genes of *Escherichia coli* and *Salmonella* spp. from fecal samples of Kafue lechwe (*Kobus leche kafuensis*) and pastoral cattle in the interface areas of Zambia. African Journal of Microbiology Research, 7, 504-508.

Lanata CF, Fischer-Walker CL, Olascoaga AC, Torres CX, Aryee MJ, Black RE, World Hlth O and Unicef, 2013. Global causes of diarrheal disease mortality in children < 5 years of age: a systematic review. PLoS One, 8.

Lang C, Prager R, Aurass P, Fruth A, Tietze E and Flieger A, 2015. Genome sequencing of two novel EHEC/EAEC hybrid strains isolated from human infections. Abstract: VTEC2015 Boston, (US), 14 Sept. 2015, p. 37. Available online: http://www.vtec2015.org/wp-content/uploads/2015/09/E-Abstracts-Oral-Poster-21.pdf.

Li L, Mendis N, Trigui H, Oliver JD and Faucher SP, 2014. The importance of the viable but non-culturable state in human bacterial pathogens. Frontiers in Microbiology, 5.

Lindsay SW, Lindsay TC, Duprez J, Hall MJR, Kwambana BA, Jawara M, Nurudeen IU, Sallah N, Wyatt N, D'Alessandro U, Pinder M and Antonio M, 2012. *Chrysomya putoria*, a putative vector of diarrheal diseases. PLoS Neglected Tropical Diseases, 6.

Mainil J and Fairbrother J, 2014. Pathogenic *Escherichia coli* in domestic mammals and birds. In: Pathogenic *Escherichia coli*, molecular and cellular microbiology. Ed Morabito S, Caister Academic Press, Norfolk, UK, 19-43.

Marietto-Goncalves GA, de Almeida SM and Rodrigues J, 2011. Presence of a human diarrheagenic *Escherichia coli* clone in captivity kept psittacidaes. The Open Microbiology Journal, 5, 72-75.

Martinez-Medina M and Garcia-Gil LJ, 2014. *Escherichia coli* in chronic inflammatory bowel diseases: An update on adherent invasive *Escherichia coli* pathogenicity. World Journal of Gastrointestinal Pathophysiology, 5, 213-227.

Maura D, Galtier M, Le Bouguenec C and Debarbieux L, 2012. Virulent bacteriophages can target O104:H4 enteroaggregative *Escherichia coli* in the mouse intestine. Antimicrobial Agents and Chemotherapy, 56, 6235-6242.

Mendez-Arancibia E, Vargas M, Soto S, Ruiz J, Kahigwa E, Schellenberg D, Urassa H, Gascon J and Vila J, 2008. Prevalence of different virulence factors and biofihn production in enteroaggregative *Escherichia coli* isolates causing diarrhea in children in Ifakara (Tanzania). American Journal of Tropical Medicine and Hygiene, 78, 985-989.

Mendez Arancibia E, Pitart C, Ruiz J, Marco F, Gascon J and Vila J, 2009. Evolution of antimicrobial resistance in enteroaggregative *Escherichia coli* and enterotoxigenic *Escherichia coli* causing traveller's diarrhoea. The Journal of Antimicrobial Chemotherapy, 64, 343-347.

Mohamed JA, Huang DB, Jiang Z-D, DuPont HL, Nataro JP, Belkind-Gerson J and Okhuysen PC, 2007. Association of putative enteroaggregative *Escherichia coli* virulence genes and biofilm production in isolates from travelers to developing countries. Journal of Clinical Microbiology, 45, 121-126.

Mohamed JA, Jiang Z, DuPont HL, Belkind-Gerson J, Tsai A, Martinez-Sandoval F, Guo DC and Okhuysen PC, 2006. A single nucleotide polymorphism (SNP) in the exon15 region of the lactoferrin gene (*LTFEx15*) is associated with travelers' diarrhea (TD). Abstract 63. Proceedings of the 44th Annual Meeting of Infectious Diseases Society of America, October 12-15, 2006, Toronto, Available online: https://idsa.confex.com/idsa/2006/webprogram/Paper22287.html

Mohlatlole RP, Madoroba E, Muchadeyi FC, Chimonyo M, Kanengoni AT and Dzomba EF, 2013. Virulence profiles of enterotoxigenic, shiga toxin and enteroaggregative *Escherichia coli* in South African pigs. Tropical Animal Health and Production, 45, 1399-1405.

Morabito S, Karch H, Mariani-Kurkdjian P, Schmidt H, Minelli F, Bingen E and Caprioli A, 1998. Enteroaggregative, shiga toxin-producing *Escherichia coli* O111:H2 associated with an outbreak of hemolytic-uremic syndrome. Journal of Clinical Microbiology, 36, 840-842.

Moran-Gilad J, Chand M, Brown C, Shetty N, Morris G, Green J, Jenkins C, Ling C, McLauchlin J, Harrison T, Goddard N, Brown K, Bolton FJ, Zambon M and Group HPAMSOP, 2012. Microbiological aspects of public health planning and preparedness for the 2012 Olympic Games. Epidemiology and Infection, 140, 2142-2151.

Muller D, Greune L, Heusipp G, Karch H, Fruth A, Tschape H and Schmidt MA, 2007. Identification of unconventional intestinal pathogenic *Escherichia coli* isolates expressing intermediate virulence factor profiles by using a novel single-step multiplex PCR. Applied and Environmental Microbiology, 73, 3380-3390.

Munera D, Ritchie JM, Hatzios SK, Bronson R, Fang G, Schadt EE, Davis BM and Waldor MK, 2014. Autotransporters but not *pAA* are critical for rabbit colonization by Shiga toxin-producing *Escherichia coli* O104:H4. Nature Communications, 5.

Nataro JP, 2005. Enteroaggregative *Escherichia coli* pathogenesis. Current Opinion in Gastroenterology, 21, 4-8.

Nataro JP, Deng YK, Deng YK and Walker K, 1994. AggR, a transcriptional activator of aggregative adherence fimbria-I expression in enteroaggregative *Escherichia coli*. Journal of Bacteriology, 176, 4691-4699.

Nataro JP and Kaper JB, 1998. Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews, 11, 142-201.

Nataro JP, Kaper JB, Robins-Browne R, Prado V, Vial P and Levine MM, 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. The Pediatric Infectious Disease Journal, 6, 829-831.

Nataro JP, Mai V, Johnson J, Blackwelder WC, Heimer R, Tirrell S, Edberg SC, Braden CR, Morris JG and Hirshon JM, 2006. Diarrheagenic *Escherichia coli* infection in Baltimore, Maryland, and New Haven, Connecticut. Clinical Infectious Diseases, 43, 402-407.

Nataro JP, Steiner T and Guerrant RL, 1998. Enteroaggregative *Escherichia coli*. Emerging Infectious Diseases, 4, 251-261.

Obi CL, Green E, Bessong PO, de Villiers B, Hoosen AA, Igumbor EO and Potgieter N, 2004. Gene encoding virulence markers among *Escherichia coli* isolates from diarrhoeic stool samples and river sources in rural Venda communities of South Africa. Water SA, 30, 37-42.

Ochoa TJ, Ruiz J, Molina M, Del Valle LJ, Vargas M, Gil AI, Ecker L, Barletta F, Hall E, Cleary TG and Lanata CF, 2009. High frequency of antimicrobial drug resistance of diarrheagenic *Escherichia coli* in infants in Peru. American Journal of Tropical Medicine and Hygiene, 81, 296-301.

Okeke IN, Lamikanra A, Czeczulin J, Dubovsky F, Kaper JB and Nataro JP, 2000. Heterogeneous virulence of enteroaggregative *Escherichia coli* strains isolated from children in southwest Nigeria. Journal of Infectious Diseases, 181, 252-260.

Okeke IN and Nataro JP, 2001. Enteroaggregative *Escherichia coli*. The Lancet Infectious Diseases, 1, 304-313.

Okeke IN, Wallace-Gadsden F, Simons HR, Matthews N, Labar AS, Hwang J and Wain J, 2010. Multi-Locus sequence typing of enteroaggregative *Escherichia coli* isolates from Nigerian children uncovers multiple lineages. PLoS One, 5.

Okhuysen PC, Jiang ZD, Carlin L, Forbes C and DuPont HL, 2004. Post-diarrhea chronic intestinal symptoms and irritable bowel syndrome in North American travelers to Mexico. American Journal of Gastroenterology, 99, 1774-1778.

Olesen B, Frimodt-Moller J, Leihof RF, Struve C, Johnston B, Hansen DS, Scheutz F, Krogfelt KA, Kuskowski MA, Clabots C and Johnson JR, 2014. Temporal trends in antimicrobial resistance and virulence-associated traits within the *Escherichia coli* sequence type 131 clonal group and its H30 and H30-Rx subclones, 1968 to 2012. Antimicrobial Agents and Chemotherapy, 58, 6886-6895.

Olesen B, Scheutz F, Andersen RL, Menard M, Boisen N, Johnston B, Hansen DS, Krogfelt KA, Nataro JP and Johnson JR, 2012. Enteroaggregative *Escherichia coli* O78: H10, the cause of an outbreak of urinary tract infection. Journal of Clinical Microbiology, 50, 3703-3711.

Oundo JO, Kariuki SM, Boga HI, Muli FW and Iijima Y, 2008. High incidence of enteroaggregative *Escherichia coli* among food handlers in three areas of Kenya: a possible transmission route of travelers' diarrhea. Journal of Travel Medicine, 15, 31-38.

Ouyang-Latimer J, Ajami NJ, Jiang ZD, Okhuysen PC, Paredes M, Flores J and DuPont HL, 2010. Biochemical and genetic diversity of enterotoxigenic *Escherichia coli* associated with diarrhea in United States students in Cuernavaca and Guadalajara, Mexico, 2004-2007. Journal of Infectious Diseases, 201, 1831-1838.

Pai M, Kang G, Ramakrishna BS, Venkataraman A and Muliyil J, 1997. An epidemic of diarrhoea in south India caused by enteroaggregative *Escherichia coli*. Indian Journal of Medical Research, 106, 7-12.

Pereira AL, Silva TN, Gomes ACMM, Araujo ACG and Giugliano LG, 2010. Diarrhea-associated biofilm formed by enteroaggregative *Escherichia coli* and aggregative *Citrobacter freundii*: a consortium mediated by putative F pili. BMC Microbiology, 10.

Perry N, Jenkins C, Cheasty T and Wain J, 2010. Diarrhoeagenic *Escherichia coli* from routine diagnostic faecal samples in England and Wales. Journal of Medical Microbiology, 59, 870-872.

PHE (Public Health England), 2013. Outbreak of *Salmonella agona* phage type 40 associated with the street spice festival, Newcastle upon Tyne February/March 2013. ILOG 8168. Available online: http://www.newcastle.gov.uk/sites/drupalncc.newcastle.gov.uk/files/wwwfileroot/environment/environmental_health/20130617_street_spice_oct_report_-_final.pdf

Philipson CW, Bassaganya-Riera J and Hontecillas R, 2013. Animal models of enteroaggregative *Escherichia coli* infection. Gut Microbes, 4, 281-291.

Pommepuy M, Butin M, Derrien A, Gourmelon M, Colwell RR and Cormier M, 1996. Retention of enteropathogenicity by viable but nonculturable *Escherichia coli* exposed to seawater and sunlight. Applied and Environmental Microbiology, 62, 4621-4626.

Prager R, Lang C, Aurass P, Fruth A, Tietze E and Flieger A, 2014. Two novel EHEC/EAEC hybrid strains isolated from human infections. PLoS One, 9.

Presterl E, Nadrchal R, Wolf D, Rotter M and Hirschl AM, 1999. Enteroaggregative and enterotoxigenic *Escherichia coli* among isolates from patients with diarrhea in Austria. European Journal of Clinical Microbiology & Infectious Diseases, 18, 209-212.

Presterl E, Zwick RH, Reichmann S, Aichelburg A, Winkler S, Kremsner PG and Graninger W, 2003. Frequency and virulence properties of diarrheagenic *Escherichia coli* in children with diarrhea in gabon. American Journal of Tropical Medicine and Hygiene, 69, 406-410.

Pritchard GC, Smith R, Ellis-Iversen J, Cheasty T and Willshaw GA, 2009. Verocytotoxigenic *Escherichia coli* O157 in animals on public amenity premises in England and Wales, 1997 to 2007. Veterinary Record, 164, 545-549.

Puno-Sarmiento J, Gazal LE, Medeiros LP, Nishio EK, Kobayashi RKT and Nakazato G, 2014. Identification of diarrheagenic *Escherichia coli* strains from avian organic fertilizers. International Journal of Environmental Research and Public Health, 11, 8924-8939.

Puno-Sarmiento J, Medeiros L, Chiconi C, Martins F, Pelayo J, Rocha S, Blanco J, Blanco M, Zanutto M, Kobayashi R and Nakazato G, 2013. Detection of diarrheagenic *Escherichia coli* strains isolated from dogs and cats in Brazil. Veterinary Microbiology, 166, 676-680.

Pupo GM, Lan RT and Reeves PR, 2000. Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. Proceedings of the National Academy of Sciences of the United States of America, 97, 10567-10572.

Qadri F, Svennerholm AM, Faruque ASG and Sack RB, 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clinical Microbiology Reviews, 18, 465-483.

Raju B and Ballal M, 2009. Multidrug resistant enteroaggregative *Escherichia coli* diarrhoea in rural southern Indian population. Scandinavian Journal of Infectious Diseases, 41, 105-108.

Rasko DA, Rosovitz MJ, Myers GSA, Mongodin EF, Fricke WF, Gajer P, Crabtree J, Sebaihia M, Thomson NR, Chaudhuri R, Henderson IR, Sperandio V and Ravel J, 2008. The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. Journal of Bacteriology, 190, 6881-6893.

Rasko DA, Webster DR, Sahl JW, Bashir A, Boisen N, Scheutz F, Paxinos EE, Sebra R, Chin CS, Iliopoulos D, Klammer A, Peluso P, Lee L, Kislyuk AO, Bullard J, Kasarskis A, Wang S, Eid J, Rank D, Redman JC, Steyert SR, Frimodt-Moller J, Struve C, Petersen AM, Krogfelt KA, Nataro JP, Schadt EE and Waldor MK, 2011. Origins of the *E. coli* strain causing an outbreak of hemolytic-uremic syndrome in Germany. New England Journal of Medicine, 365, 709-717.

Ren CP, Chaudhuri RR, Fivian A, Bailey CM, Antonio M, Barnes WA and Pallen MJ, 2004. The ETT2 gene cluster, encoding a second type III secretion system from *Escherichia coli,* is present in the majority of strains but has undergone widespread mutational attrition. Journal of Bacteriology, 186, 3547-3560.

Roche JK, Cabel A, Sevilleja J, Nataro J and Guerrant RL, 2010. Enteroaggregative *Escherichia coli* (EAEC) impairs growth while malnutrition worsens EAEC infection: a novel murine model of the infection malnutrition cycle. Journal of Infectious Diseases, 202, 506-514.

Roest HIJ, Liebana E, Wannet W, van Duynhoven Y, Veldman KT and Mevius DJ, 2007. Antibiotic resistance in *Escherichia coli* O157 isolated between 1998 and 2003 in the Netherlands. Tijdschrift Voor Diergeneeskunde, 132, 954-958.

Rosenwasser LJ and Borish L, 1998. Promoter polymorphisms predisposing to the development of asthma and atopy. Clinical and Experimental Allergy, 28, 13-15.

Russell AB, Peterson SB and Mougous JD, 2014. Type VI secretion system effectors: poisons with a purpose. Nature Reviews Microbiology, 12, 137-148.

Ruttler ME, Renna NF, Balbi L, Garcia B, Guidone L, Fernandez R, Puig O and Ortiz A, 2002. Characterization of enteroaggregative *Escherichia coli* strains isolated from children with acute diarrhea, in Mendoza, Argentina. Revista Argentina de Microbiologia, 34, 167-170.

Saha DR, Guin S, Krishnan R, Nag D, Koley H, Shinoda S and Ramamurthy T, 2013. Inflammatory diarrhea due to enteroaggregative *Escherichia coli*: evidence from clinical and mice model studies. Gut Pathogens, 5.

Sahl JW, Morris CR, Emberger J, Fraser CM, Ochieng JB, Juma J, Fields B, Breiman RF, Gilmour M, Nataro JP and Rasko DA, 2015. Defining the phylogenomics of *Shigella* species: a pathway to diagnostics. Journal of Clinical Microbiology, 53, 951-960.

Sainz T, Perez J, Fresan MC, Flores V, Jimenez L, Hernandez U, Herrera I and Eslava C, 2002. Histological alterations and immune response induced by pet toxin during colonization with enteroaggregative *Escherichia coli* (EAEC) in a mouse model infection. Journal of Microbiology, 40, 91-97.

Sakkejha H, Byrne L, Lawson AJ and Jenkins C, 2013. An update on the microbiology and epidemiology of enteropathogenic *Escherichia coli* in England 2010-2012. Journal of Medical Microbiology, 62, 1531-1534.

Sang WK, Oundo JO, Mwituria JK, Waiyaki PG, Yoh M, Iida T and Honda T, 1997. Multidrug-resistant enteroaggregative *Escherichia coli* associated with persistent diarrhea in Kenyan children. Emerging Infectious Diseases, 3, 373-374.

Santiago AE, Ruiz-Perez F, Jo NY, Vijayakumar V, Gong MQ and Nataro JP, 2014. A large family of antivirulence regulators modulates the effects of transcriptional activators in Gram-negative pathogenic bacteria. PLoS Pathogens, 10.

Sarantuya J, Nishi J, Wakimoto N, Erdene S, Nataro JP, Sheikh J, Iwashita M, Manago K, Tokuda K, Yoshinaga M, Miyata K and Kawano Y, 2004. Typical enteroaggregative *Escherichia coli* is the most prevalent pathotype among *E. coli* strains causing diarrhea in Mongolian children. Journal of Clinical Microbiology, 42, 133-139.

Sasaki Y, Usui M, Murakami M, Haruna M, Kojima A, Asai T and Yamada Y, 2012. Antimicrobial resistance in Shiga toxin-producing *Escherichia coli* O157 and O26 isolates from beef cattle. Japanese Journal of Infectious Diseases, 65, 117-121.

Scavia G, Morabito S, Tozzoli R, Michelacci V, Marziano ML, Minelli F, Ferreri C, Paglialonga F, Edefonti A and Caprioli A, 2011. Similarity of Shiga toxin-producing *Escherichia coli* O104:H4 strains from Italy and Germany. Emerging Infectious Diseases, 17, 1957-1958.

Scavia G, Staffolani M, Fisichella S, Striano G, Colletta S, Ferri G, Escher M, Minelli F and Caprioli A, 2008. Enteroaggregative *Escherichia coli* associated with a foodborne outbreak of gastroenteritis. Journal of Medical Microbiology, 57, 1141-1146.

Scheutz F, Espenhain L, Ethelberg S, Kjelsø C, Mølbak K, Olsen KEP, Petersen AM, Olesen B, Engberg J, Holt HM, Friis-Møller A, Lemming L, Schønning Rosenvinge F, Prag J, Jensen TE, Tvede M and Kjældgaard P, 2014. Diarréfremkaldende *E.coli* 2000-2012. EPI - Nyt, 10, 1. Available online: http://www.ssi.dk/Aktuelt/Nyhedsbreve/EPI-NYT/2014/Uge%2010%2020-%202014.aspx

Scheutz F, Nielsen EM, Frimodt-Moller J, Boisen N, Morabito S, Tozzoli R, Nataro JP and Caprioli A, 2011. Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011. Eurosurveillance, 16, 1-6.

Schmidt H, Knop C, Franke S, Aleksic S, Heesemann J and Karch H, 1995. Development of PCR for screening of enteroaggregative *Escherichia coli*. Journal of Clinical Microbiology, 33, 701-705.

Seifert ME and Tarr PI, 2012. Azithromycin decolonization of STEC-a new risk emerges. Nature Reviews Nephrology, 8.

Shah N, DuPont HL and Ramsey DJ, 2009. Global etiology of Travelers' Diarrhea: systematic review from 1973 to the present. American Journal of Tropical Medicine and Hygiene, 80, 609-614.

Sheikh J, Czeczulin JR, Harrington S, Hicks S, Henderson IR, Le Bouguenec C, Gounon P, Phillips A and Nataro JP, 2002. A novel dispersin protein in enteroaggregative *Escherichia coli*. The Journal of Clinical Investigation, 110, 1329-1337.

Sheikh J, Dudley EG, Sui BQ, Tamboura B, Suleman A and Nataro JP, 2006. EilA, a HilA-like regulator in enteroaggregative *Escherichia coli*. Molecular Microbiology, 61, 338-350.

Sheikh J, Hicks S, Dall'Agnol M, Phillips AD and Nataro JP, 2001. Roles for *Fis* and *YafK* in biofilm formation by enteroaggregative *Escherichia coli*. Molecular Microbiology, 41, 983-997.

Shin J, Oh SS, Oh KH, Park JH, Jang EJ, Chung GT, Yoo CK, Bae GR and Cho SH, 2015. An outbreak of foodborne illness caused by enteroaggregative *Escherichia coli* in a high school, South Korea. Japanese Journal of Infectious Diseases, 68, 514-519.

Sidhu JPS, Ahmed W, Hodgers L and Toze S, 2013. Occurrence of virulence genes associated with diarrheagenic pathotypes in *Escherichia coli* isolates from surface water. Applied and Environmental Microbiology, 79, 328-335.

Smith HR, Cheasty T and Rowe B, 1997. Enteroaggregative *Escherichia coli* and outbreaks of gastroenteritis in UK. Lancet, 350, 814-815.

Snelling AM, Macfarlane-Smith LR, Fletcher JN and Okeke IN, 2009. The commonly-used DNA probe for diffusely-adherent *Escherichia coli* cross-reacts with a subset of enteroaggregative *E. coli*. BMC Microbiology, 9.

Sobieszczanska B, Kowalska-Krochmal B, Mowszet K and Pytrus T, 2003. Susceptibility to antimicrobial agents of enteroaggregative *Escherichia coli* strains isolated from children with diarrhea. Przeglad Epidemiologiczny, 57, 499-503.

Soon JM, Seaman P and Baines RN, 2013. *Escherichia coli* O104:H4 outbreak from sprouted seeds. International Journal of Hygiene and Environmental Health, 216, 346-354.

Spina A, Kerr KG, Cormican M, Barbut F, Eigentler A, Zerva L, Tassios P, Popescu GA, Rafila A, Eerola E, Batista J, Maass M, Aschbacher R, Olsen KEP and Allerberger F, 2015. Spectrum of enteropathogens detected by the FilmArray GI Panel in a multicentre study of community-acquired gastroenteritis. Clinical Microbiology and Infection, 21, 719-728.

Subodh P, Mandal PS, Samanta I, Joardar SN and Isore DP, 2013. Association of enteroaggregative heat stable enterotoxin gene (*east*1) possessing *Escherichia coli* with duck mortality. Indian Journal of Animal Health, 52, 57-58.

Tam CC, O'Brien SJ, Tompkins DS, Bolton FJ, Berry L, Dodds J, Choudhury D, Halstead F, Iturriza-Gomara M, Mather K, Rait G, Ridge A, Rodrigues LC, Wain J, Wood B, Gray JJ and Comm IIDSE, 2012a. Changes in causes of acute gastroenteritis in the United Kingdom over 15 years: microbiologic findings from 2 prospective, population-based studies of infectious intestinal disease. Clinical Infectious Diseases, 54, 1275-1286.

Tam CC, Rodrigues LC, Viviani L, Dodds JP, Evans MR, Hunter PR, Gray JJ, Letley LH, Rait G, Tompkins DS, O'Brien SJ and Comm IIDSE, 2012b. Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. Gut, 61, 69-77.

Thabane M, Kottachchi DT and Marshall JK, 2007. Systematic review and meta-analysis: the incidence and prognosis of post-infectious irritable bowel syndrome. Alimentary Pharmacology & Therapeutics, 26, 535-544.

Threlfall J, Ward LR, Frost JA, Cheasty T and Willshaw GA, 1999. The emergence and spread of antibiotic resistance in food-brone bacteria in the United Kingdom. APUA Newsletter 17, 1-7.

Tietze E, Dabrowski PW, Prager R, Radonic A, Fruth A, Aurass P, Nitsche A, Mielke M and Flieger A, 2015. Comparative genomic analysis of two novel sporadic Shiga toxin-producing *Escherichia coli* O104:H4 strains isolated 2011 in Germany. PLoS One, 10.

Toma C, Lu Y, Higa N, Nakasone N, Chinen I, Baschkier A, Rivas M and Iwanaga M, 2003. Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. Journal of Clinical Microbiology, 41, 2669-2671.

Tozzoli R, Grande L, Michelacci V, Ranieri P, Maugliani A, Caprioli A and Morabito S, 2014. Shiga toxin-converting phages and the emergence of new pathogenic *Escherichia coli*: a world in motion. Frontiers in Cellular and Infection Microbiology, 4, 80.

Tozzoli R and Scheutz F, 2014. Diarrhoeagenic *Escherichia coli* infections in humans. In: Pathogenic *Escherichia coli*: molecular and cellular microbiology. Ed Morabito S, Caister Academic Press, Norfolk, UK, 1-18.

Tremblay YDN, Hathroubi S and Jacques M, 2014. The bacterian biofilms - their importance in animal health and in public health -Yannick DN Tremblay. Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire, 78, 110-116.

Trivenee T, Shubhangi W and Dewanand K, 2012. Prevalence of EPEC and EAEC virulence genes among bovine and environmental *E. coli*. Proceedings of the 12th Indian Veterinary Congress and XIX Annual Conference of Indian Association for the Advancement of Veterinary Research, 20-21 April, 2012, Mhow, Madhya Pradesh, India, 2012, 81-81.

Tsai CC, Chen SY and Tsen HY, 2003. Screening the enteroaggregative *Escherichia coli* activity and detection of the *aggA*, *aafA*, and *astA* genes with novel PCR primers for the *Escherichia coli* isolates from diarrhea cases in Taiwan. Diagnostic Microbiology and Infectious Disease, 46, 159-165.

Uber AP, Trabulsi LR, Irino K, Beutin L, Ghilardi ACR, Gomes TAT, Liberatore AMA, de Castro A and Elias WP, 2006. Enteroaggregative *Escherichia coli* from humans and animals differ in major phenotypical traits and virulence genes. FEMS Microbiology Letters, 256, 251-257.

Valencia-Gallardo CM, Carayol N and Nhieu GTV, 2015. Cytoskeletal mechanics during *Shigella* invasion and dissemination in epithelial cells. Cellular Microbiology, 17, 174-182.

van den Beld MJC and Reubsaet FAG, 2012. Differentiation between *Shigella*, enteroinvasive *Escherichia coli* (EIEC) and noninvasive *Escherichia coli*. European Journal of Clinical Microbiology & Infectious Diseases, 31, 899-904.

Vasudevan P, Annamalai T, Sartori L, Hoagland T and Venkitanarayanan K, 2003. Behavior of enteroaggregative *Escherichia coli* in bottled spring and mineral water. Journal of Food Protection, 66, 497-500.

Veilleux S and Dubreuil JD, 2006. Presence of *Escherichia coli* carrying the *EAST1* toxin gene in farm animals. Veterinary Research, 37, 3-13.

Vial PA, Robins-Browne R, Lior H, Prado V, Kaper JB, Nataro JP, Maneval D, Elsayed A and Levine MM, 1988. Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent of diarrheal disease. The Journal of Infectious Diseases, 158, 70-79.

Vila J, Vargas M, Ruiz J, Espasa M, Pujol M, Corachan M, De Anta MTJ and Gascon J, 2001. Susceptibility patterns of enteroaggregative *Escherichia coli* associated with traveller's diarrhoea: emergence of quinolone resistance. Journal of Medical Microbiology, 50, 996-1000.

Wakimoto N, Nishi J, Sheikh J, Nataro JP, Sarantuya J, Iwashita M, Manago K, Tokuda K, Yoshinaga M and Kawano Y, 2004. Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative *Escherichia coli*. American Journal of Tropical Medicine and Hygiene, 71, 687-690.

Wallace-Gadsden F, Johnson JR, Wain J and Okeke IN, 2007. Enteroaggregative *Escherichia coli* related to uropathogenic clonal group A. Emerging Infectious Diseases, 13, 757-760.

Wani SA, Hussain I, Beg SA, Rather MA, Kabli ZA, Mir MA and Nishikawa Y, 2013. Diarrhoeagenic *Escherichia coli* and salmonellae in calves and lambs in Kashmir: absence, prevalence and antibiogram. Revue Scientifique et Technique-Office International des Epizooties, 32, 833-840.

Wani SA, Hussain I, Rather MA, Kabli ZA, Nagamani K, Nishikawa Y, Qureshi SD and Khan I, 2012. Putative virulence genes and biofilm production among typical enteroaggregative *Escherichia coli* isolates from diarrhoeic children in Kashmir and Andhra Pradesh. Indian Journal of Microbiology, 52, 587-592.

Wanke CA, Mayer H, Weber R, Zbinden R, Watson DA and Acheson D, 1998. Enteroaggregative *Escherichia coli* as a potential cause of diarrheal disease in adults infected with human immunodeficiency virus. Journal of Infectious Diseases, 178, 185-190.

Weiner M, Dacko J and Osek J, 2004. Molecular analysis of enterotoxigenic, shigatoxigenic and enteroaggregative *Escherichia coli* strains isolated from suckling piglets with diarrhoea by the use of pulsed-field electrophoresis. Bulletin of the Veterinary Institute in Pulawy, 48, 225-231.

Welch RA, Burland V, Plunkett G, Redford P, Roesch P, Rasko D, Buckles EL, Liou SR, Boutin A, Hackett J, Stroud D, Mayhew GF, Rose DJ, Zhou S, Schwartz DC, Perna NT, Mobley HLT, Donnenberg MS and Blattner FR, 2002. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America, 99, 17020-17024.

Wieler LH, Semmler T, Eichhorn I, Antao EM, Kinnemann B, Geue L, Karch H, Guenther S and Bethe A, 2011. No evidence of the Shiga toxin-producing *E. coli* O104:H4 outbreak strain or enteroaggregative *E. coli* (EAEC) found in cattle faeces in northern Germany, the hotspot of the 2011 HUS outbreak area. Gut Pathogens, 3.

Wilson A, Evans J, Chart H, Cheasty T, Wheeler JG, Tompkins D and Smith HR, 2001. Characterisation of strains of enteroaggregative *Escherichia coli* isolated during the infectious intestinal disease study in England. European Journal of Epidemiology, 17, 1125-1130.

Wu GH, Day MJ, Mafura MT, Nunez-Garcia J, Fenner JJ, Sharma M, van Essen-Zandbergen A, Rodriguez I, Dierikx C, Kadlec K, Schink AK, Wain J, Helmuth R, Guerra B, Schwarz S, Threlfall J, Woodward MJ, Woodford N, Coldham N and Mevius D, 2013. Comparative analysis of ESBL-positive *Escherichia coli* isolates from animals and humans from the UK, the Netherlands and Germany. PLoS One, 8.

Yamaichi Y, Chao MC, Sasabe J, Clark L, Davis BM, Yamamoto N, Mori H, Kurokawa K and Waldor MK, 2014. High-resolution genetic analysis of the requirements for horizontal transmission of the ESBL plasmid from *Escherichia coli* O104:H4. Nucleic Acids Research, 1–13.

Yamamoto T, Echeverria P and Yokota T, 1992. Drug-resistance and adherence to human intestines of enteroaggregative *Escherichia coli*. Journal of Infectious Diseases, 165, 744-749.

Zangari T, Melton-Celsa AR, Panda A, Boisen N, Smith MA, Tatarov I, De Tolla LJ, Nataro JP and O'Brien AD, 2013. Virulence of the Shiga toxin Type 2-Expressing *Escherichia coli* O104:H4 German outbreak isolate in two animal models. Infection and Immunity, 81, 1562-1574.

Zurfluh K, Nuesch-Inderbinen M, Morach M, Zihler Berner A, Hachler H and Stephan R, 2015. Extended-spectrum-beta-lactamase-producing Enterobacteriaceae isolated from vegetables imported from the Dominican Republic, India, Thailand, and Vietnam. Applied and Environmental Microbiology, 81, 3115-3120.

Glossary and Abbreviations

	Pathogenic *Escherichia coli*
AIEC	Adherent invasive *Escherichia coli*
DAEC	Diffusely Adherent *E. coli*
EaggEC	Enteroaggregative *E. coli* (now referred to as EAEC – see below)
EAEC	Enteroaggregative *E. coli* (formerly known as EaggEC – see above)
EHEAC	Enteroaggregative Haemorrhagic *E. coli*
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extra-intestinal pathogenic *E. coli*
NMEC	Neonatal meningitis *E. coli*
SePEC	Septicaemic pathogenic *E. coli*
STEC	Shiga toxin-producing *E. coli* (synonymous with VTEC, see below)
UPEC	Uropathogenic *E. coli*
VTEC	Verocytotoxin-producing *E. coli*, also known as verotoxigenic *E. coli*, verocytotoxigenic *E. coli,* verotoxin-producing *E. coli* and Shiga toxin-producing *E. coli* (STEC) (see above)

	Relevant *E. coli* terminology
Serogroup	Classification of *E. coli* strains based on identification of 'O' (lipopolysaccharide) antigen – e.g. O157, O104
Serotype	Classification of *E. coli* strains based on identification of 'O' (lipopolysaccharide) and 'H' (flagella) antigen – e.g. O157:H7; O104:H4

	Virulence-associated factors
AAF	Aggregative adherence fimbriae
AAF/1	Aggregative adherence fimbriae type 1
AggR	Plasmid-encoded transcriptional regulator mediating expression of AAF (see above)
BFP	Bundle forming pilus
CFA	Colonisation factor antigen(s)
EAF	EPEC Adherence factor (plasmid)
EAST1	Enteroaggregative heat-stable toxin often produced by EAEC (see above)
EspC,F, H	Effector proteins encoded by Locus of enterocyte effacement (LEE) – see below
EspP	*E. coli*-secreted protein (see SPATE)
ETT2	Potentially functional but as yet uncharacterised Type III secretion system
HlyE	Haemolysin type E
Intimin	Effector protein encoded by LEE – see below

Ipa	Invasion plasmid antigen(s)
IpaH3	Invasion plasmid antigen H3
IRP	Iron regulatory protein
LEE	Locus of enterocyte effacement. A moderately conserved 35,000 bp pathogenicity island in the *E. coli* genome. The LEE encodes the Type III secretion system and associated chaperones and effector proteins responsible for AE lesions in the large intestine. These proteins include intimin, Tir, EspC, EspF, EspH, and Map protein (see above and below).
LPF	Long polar fimbriae
Map	Effector protein encoded by LEE (see above)
MIC	Minimum Inhibitory Concentration
PAI	Pathogenicity island
Pet	Plasmid-encoded toxin (see SPATE)
Pic	Protein involved in intestinal colonisation (See SPATE)
Saa	STEC autoagglutinating adhesion
Sat	Sectreted autotransporter toxin (see SPATE)
SepA	*Shigella* SPATE toxin
ShET	*Shigella* enterotoxin
Shf	Protein thought to be involved in intracellular adhesion
SigA	*Shigella* IgA-like protease homology (see SPATE)
SPATE	Serine protease autotransporters of *Enterobacteriaceae*
Stx	Shiga toxin
Stx1,2	Shiga toxin types 1, 2
T3SS	Type III secretion system
T6SS	Type VI secretion system
Tia	Toxigenic invasion locus A
Tir	Effector protein encoded by LEE (see above)
VT	Vero cytotoxin (synonymous with ST - see above)

Virulence-associated genes

aaiC	Chromosomally-encoded gene encoding secreted protein of enteroaggregative *Escherichia coli* (EAEC)
aagR	Plasmid-encoded regulator gene
aar	Gene encoding the novel EAEC regulator Aar, an ArrR-regulated activator
ast	Plasmid-encoded gene responsible for production of heat-stable enterotoxin EAST1 (see above)
eae	Intimin-encoding gene of Shiga toxin-producing *E. coli*
hly	Gene encoding production of haemolysin
ipa	Operon encoding invasion plasmid antigen(s)
irp	Gene encoding production of an iron regulatory protein
shf	Gene encoding the Shf protein (see above)

stx1, stx2, stx3	Shiga toxin-encoding genes
yafK	Gene encoding protein required for transcription of AAF/II-encoding genes

Note: Full details of virulence-associated factors in EAEC and virulence genes therein are provided in Tables 2 and 3 in the body text.

Abbreviations

AA	Aggregative adherence
AE	Attaching and effacing
AMR	Antimicrobial resistance
BIOHAZ	EFSA Biological Hazards Panel
Bp	Base pair(s)
CD	Crohn's disease
CF	Colonisation factor(s)
cfu	Colony forming units
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EFSA	European Food Safety Authority
EQA	External Quality Assessment
ESBL	Extended Spectrum Beta(β)-Lactamase
EU	European Union
EU RL	European Union Reference Laboratory
FSA	Food Standards Agency (England and Wales)
FWD	Food and Waterborne Diseases (network) [ECDC network]
GEMS	Global Enteric Multicenter Study
GI	Gastrointestinal
HC	Haemorrhagic colitis
HeLa	HeLa cell line
Hep 2	Human Epithelial type 2 (cells)
HUS	Haemolytic Uraemic Syndrome
IBS	Irritable Bowel Syndrome
IID	Infectious Intestinal Disease (in the Community) [UK studies in 1993-96 and 2008-09]
IL	Interleukin
Inc	Incompatibility group
kb	Kilobase
LPS	Lipopolysaccharide
LT	Heat-labile

Mda	Megadalton(s)
MLST	Multi Locus Sequence Type(s) / Typing
ND	Not determined
NM	Non-motile
NMR	Nuclear Magnetic Resonance
NRL	National Reference Laboratory
NT	Not tested/unknown
OMV(s)	Outer Membrane Vesicle(s)
PCR	Polymerase chain reaction
PHE	Public Health England
PT	Proficiency test(s)
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SNP	Single nucleotide polymorphism
ST	Heat-stable
spp.	Species
ST	Sequence type
Stx	Shiga toxin
TESSy	The European Epidemiological Surveillance System
ToR(s)	Term(s) of reference
UK	United Kingdom
USA	United States of America
UTI(s)	Urinary Tract Infection(s)
VBNC	Viable but non-culturable
WG	Work Group
wgMLST	whole genome MLST
WGS	Whole genome sequencing
WoK	Web of Knowledge

Antimicrobial resistance symbols

A	Ampicillin
C	Chloramphenicol
S	Streptomycin
Su	Sulphonamides
T	Tetracyclines
Tp	Trimethoprim

Appendix A – Questionnaire on the occurrence of EAEC in food, feed and animals

EAEC Questionnaire

Food/Feed/Animal samples

Many thanks for collaborating with the BIOHAZ WG on the public health risks associated with Enteroaggregative *Escherichia coli* (EAEC) as a foodborne pathogen (EFSA-Q-2014-00536) by providing answers to the questions indicated below.

Please check the relevant multiple choice options and fill in this questionnaire according to the available information in your country.

1. Do you have implemented a method for the detection of EAEC in food / feed / animal samples?
   ☐ EU RL VTEC method (http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_05_Rev_1.pdf)
   ☐ PCR If so, please specify amplified genes: _____
   ☐ Bacterial Culture
   ☐ Cell culture
   ☐ Other methods If so, please specify which: _____

2. If so, do you have accredited a method for the detection of EAEC in food/feed/animal samples?
   ☐ EU RL VTEC method (http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_05_Rev_1.pdf)
   ☐ Other methods If so, please specify which: _____

3. Do you search for EAEC in food/feed/animal samples? If so, please fill in the table below:

Year	Official **food** samples		Other **food** samples (industry, HACCP)	
	Number of tested samples	Number of positive samples	Number of tested samples	Number of positive samples
2012				
2013				
2014				

Year	Official **feed** samples		Other **feed** samples (industry, HACCP)	
	Number of tested samples	Number of positive samples	Number of tested samples	Number of positive samples
2012				
2013				
2014				

Year	Official **animal** samples		Other **animal** samples (industry, HACCP)	
	Number of tested samples	Number of positive samples	Number of tested samples	Number of positive samples
2012				
2013				
2014				

4. Please fill in the table below if there is the relevant detailed information on the food matrix available in your country.

	If appropriate, number of samples analysed/food matrix/year		
	2012	2013	2014
Food matrix 1 (please specify)			
Food matrix 2 (please specify)			
…			
…			

5. If appropriate, do you perform any additional characterisation of EAEC isolates in food/feed/animals (e.g. serotype, AMR profile etc.)?

_____
_____
_____
_____

6. Are any data on the occurrence of EAEC in food/feed animals published in official monitoring reports in your country?
   ☐ Yes
   ☐ No
   If so, can you please provide details of such reports and how they may be accessed?

_____
_____
_____
_____

7. Are you aware of any additional EAEC data other than the official monitoring (e.g. research activities) in your country?
   ☐ Yes
   ☐ No
   If so, please provide any available details.

_____
_____
_____
_____

Appendix B – Summary of the replies provided by Member States to the questionnaire on EAEC in food, feed and animals

The questionnaire was sent to the 31 countries in the Scientific Network for Zoonoses Monitoring Data.

Number of countries that replied: 23 EU/EEA countries; response rate 74,1%

(BE, CH, CR, CZ, DE, DK, EE, ES, FI, FR, GR, HU, IE, IT, LT, LV, NL, NO, PL, PT, SE, SI, SK)

Methods used for the detection of EAEC in food/feed/animal samples (in 20 countries that have a method for detection of EAEC):

20/23 (86,9%) countries have implemented a method for the detection of EAEC in food/feed/animal samples. Some comments:

- EU RL VTEC method (17 countries)
- PCR (*aggR*, *aaiC*) (4 countries)
- PCR (*aggR,* wzxO104, fliCH4) (1 country)
- Bacterial culture (2 countries)
- Use of ISO 13136 for enrichment and use the PCR tests specified in the EU RL VTEC method 05 rev1 for testing of enrichment broths and isolates (1 country)
- EU RL VTEC method NRL feed animals (CODA - CERVA)/NRL food (WIV-ISP, Scientific Public Health Institute), validated method available for detection of aaiC genes and aggR genes on isolates and on enrichment medium, developed by a scientific project (1 country):
  References a*ggR* genes: Fukushima H, Katsube K, Tsunomori Y, Kishi R, Atsuta J and Akiba Y, 2009. Comprehensive and rapid real-time PCR analysis of 21 foodborne outbreaks. International Journal of Microbiology, 917623.
  References *aaiC* genes: Boisen N, Scehutz F, Rasko DA, redman JC, Persson S, Simon J, Kotloff KL, Levine MM, Sow S, Tamboura B, Toure A, Malle D, Panchalingam S, Krogfelt KA and Nataro JP, 2012. Genomic characterization of enteroaggregative *Escherichia coli* from children in Mali. Journal of Infectious Diseases, 205, 431–444.
- No (1 country)
- Empty reply (1 country)

Some countries use more than one method.

Accreditation of a method for the detection of EAEC in food/feed/animal samples:

No country has an accredited method for the detection of EAEC in food/feed/animal samples. Some comments:

- Not accredited (6 countries)
- Empty reply (13 countries)
- Not yet (1 country)
- The NRL foresees the accreditation of the EU RL VTEC method for the year 2016 (1 country)
- NRL food (WIV-ISP, Scientific Public Health Institute), validated method available for detection of *aaiC* genes and *aggR* genes on isolates and on enrichment medium, developed by a scientific project (1 country)

Summary of the collected answers on numbers of samples tested for EAEC and positive food/feed/animal samples:

Year	Official food samples		Other food samples (industry, HACCP)	
	Total number of tested samples	Total number of positive samples	Total number of tested samples	Total number of positive samples
2012				
2013	601 (SK, PT, CZ)	0 (SK, PT, CZ)	21 (SK) unknown (FR)[(a)]	2 (SK)
2014	3,099 (NL, FR, SK, PT, CZ)	0 (NL, FR, SK, PT, CZ)	176 (SK, CH), unknown (FR) 14 (ES: did not provide number of positive samples)	2 (SK, CH)

CH: Switzerland; CZ: Czech Republic; ES: Spain; FR: France; HACCP: Hazard Analysis and Critical Control Points; NL: the Netherlands; PT: Portugal; SK: Slovakia.
(a): FR: In 2015, 189 food matrices will be screened for presence of EAEC.

No information was provided by any country regarding feed samples.

Year	Official animal samples		Other animal samples (industry, HACCP)	
	Total number of tested samples	Total number of positive samples	Total number of tested samples	Total number of positive samples
2012	66 pooled samples (bovine faeces) (ES)	8 pooled samples PCR positive to *aggR* (ES)	2,500 (SE) (2,000 faecal samples/500 ear samples from a slaughterhouse prevalence study)	0 (SE)
2013				
2014	250 (NL)	0 (NL)	unknown (FR)	

ES: Spain; FR: France; HACCP: Hazard Analysis and Critical Control Points; NL: the Netherlands; PCR: Polymerase Chain Reaction; SE: Sweden.

If appropriate, total number of samples analysed/food matrix/year	2012	2013	2014
Vegetables (including sprouts)			580 (NL) 169 (CH)
Sprouts		5 (SK)	1 (SK)
Batches of sprouts			8 (ES)
Irrigation water of sprouts			6 (ES)
Salad		3 (SK)	
Vegetable salad/ prepared meal		1 (SK)	4 (SK)
Chicken meat/ mixed salad with chicken		1 (SK)	1 (SK)
Meat (raw meat and meat preparations)			1,950 (NL)
Cheeses (raw milk)			151 (NL, FR)

CH: Switzerland; ES: Spain; FR: France; NL: the Netherlands; SK: Slovakia.

Performing of any additional characterisation of EAEC isolates in food/feed/animals (e.g. serotype, AMR profile etc.). Some comments:

- None, no additional confirmation or empty reply (9 countries)
- Yes, serotyping, PFGE (2 countries)
- Yes, for all isolates sent to the National Reference Laboratory (besides commensal *E.coli* specifically collected for antimicrobial resistance testing) we would perform serotyping AMR profile, testing for virulence genes.
- Not relevant. If we found EAEC strains we would probably use WGS for characterization purposes.
- Not relevant as no EAEC strains have been isolated; however if strains would have been isolated, additional characterisation would have been: serotyping, AMR profile, PFGE typing at least.

- In animals no isolates were obtained from the positive samples. Gene copy number was really low for *aggR* target.
- Our Lab is capable of Serotyping (PCR for the 15 most frequent VTEC serotypes), and test for AMR profile of EAEC isolates.
- We have already introduced the method in our laboratory but for further testing of STEC and not for direct detection of EAEC. It means that we would perform the test if *stx1* or *stx2* genes would be identified without the presence of *eae* gene. Until 2014, only sprouts were tested for *stx1* and *stx2*, but there were no positives without *eae* genes to be further tested to EAEC genes (a*ggR* and *aaiC*). If we would find, we can test the isolate further by serotyping, AMR or PFGE.
- If available, the strains can be characterized by serotyping and WGS.
- In such a situation, we would most likely carry out serotyping, analyse for other virulence factors and if more than one isolated were obtained, PFGE. In the current situation we would most likely only analyse for EAEC in outbreak situations. If an isolate is obtained this would be sent to the National Institute for Public Health for comparison with the outbreak strain.
- AMR profile and serotype 104 possible to define.
- *E. coli* phylogroup typing; MLST typing; results 2014: EAEC D:ST38 ESBL producer.

Are any data on the occurrence of EAEC in food/feed animals published in official monitoring reports in your country?

- All countries: No or empty reply.

Are you aware of any additional EAEC data other than the official monitoring (e.g. research activities) in your country?

- No (14 countries)
- DE:
    - Wieler LH, Semmler T, Eichhorn I, Antao EM, Kinnemann B, Geue L, Karch H, Guenther S, Bethe A, 2011. No evidence of the Shiga toxin-producing *E. coli* O104:H4 outbreak strain or enteroaggregative *E. coli* (EAEC) found in cattle faeces in northern Germany, the hotspot of the 2011 HUS outbreak area. Gut Pathogy, 3(1),17. doi: 10.1186/1757-4749-3-17.
- FR:
    - Data from research activities conducted in bovine and slaughterhouses effluent in France. Bibbal D, Kérourédan M, Loukiadis E, Scheutz F, Oswald E, Brugère H, 2014. Slaughterhouse effluent discharges into rivers not responsible for environmental occurrence of enteroaggregative *Escherichia coli*. Veterinary Microbiology, 168, 451–454.
    - A total of 10,618 *E. coli* isolates were screened by PCR for the presence of EAEC-associated genetic markers (*aggR*, *aap* and *aatA*). None of these markers was detected in *E. coli* isolated from slaughterhouse samples. A unique enteroaggregative *E. coli* (EAEC) O126:H8 was detected in river water sampled upstream from slaughterhouse effluent discharge. These results confirmed that animals might not be reservoirs of EAEC, and that further studies are required to evaluate the role of the environment in the transmission of EAEC to humans.
    - Auvray F, Dilasser F, Bibbal D, Kérourédan M, Oswald E, Brugère H, 2012. French cattle is not a reservoir of the highly virulent enteroaggregative Shiga toxin-producing *Escherichia coli* of serotype O104:H4. Veterinary Microbiology, 158, 443–445.
      A total of 1,468 French cattle were analysed for faecal carriage of the outbreak strain by PCR assays targeting *stx2*, *wzxO104*, *fliCH4* and *aggR* genetic markers. None of the faecal samples contained the four markers simultaneously, indicating that cattle are not a reservoir of this recently emerged *E. coli* pathotype.
- NL:
    - National Institute of Public Health (RIVM) is carrying out research into the virulence factors of Shiga-toxin producing *E.coli*. This is done with financial support of the NVWA. Research is still going on.

- RIVM conducted a study into the environmental persistence of Stx-producing and non-producing EAEC O104. Publication is in progress. Persistence of these strains is comparable with O157, and strongly depends on the general stress gene *rpoS*.

- IT:
  - A specific survey on the presence of EAEC in farm animals has been carried out in the framework of an ongoing research project supported by the Ministry of Health. The samples tested were fecal pool collected from bovine (116), ovine (97), bufaline (34) and pig (89) farms. The EU RL VTEC method was applied to fecal samples. All the samples tested were negative.
- CH:
  - Nüesch-Inderbinen et al., 2013. Characteristics of enteroaggregative *Escherichia coli* isolated from healthy carriers and from patients with diarrhea. Journal of Medical Microbiology, 62, 1828-1834.
  - Nüesch-Inderbinen et al., 2013. Cross-sectional study on fecal carriage of Enterobacteriaceae with resistance to extended-spectrum cephalosporins in primary care patients. Microbial Drug Resistance, 19, 362-9.
  - Zurfluh K, Nüesch-Inderbinen M, Morach M, Zihler Berner A, Hachler H amd Stephan R, 2015. Extended-spectrum ß-lactamase-producing-Enterobacteriaceae in vegetables imported from the Dominican Republic, India, Thailand and Vietnam. Applied and Environmental Microbiology, 81, 3115–3120.
  - Müller et al., in press. Distribution of virulence factors in ESBL-producing *Escherichia coli* isolated at the environment, livestock, food and human interface. Science of the Total Environment, submitted.
  - Occurrence of EAEC in fecal samples of slaughtered cattle in Switzerland. Master thesis, ongoing.

Appendix C – Questionnaire on the occurrence of EAEC in humans

Enteroaggregative Escherichia coli (EAEC) Questionnaire

Fields marked with * are mandatory.

ecdc
EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL

efsa
European Food Safety Authority

1

EFSA BIOHAZ panel has established a working group (WG) to assess the public health risks associated with Enteroaggregative Escherichia coli (EAEC) as a foodborne pathogen (EFSA-Q-2014-00536). ECDC is participating in the WG, which has drafted a brief questionnaire in order to gather available data on the EAEC in humans as well as on the used microbiological diagnostic methods.

We kindly ask you to reply to nine questions below if you have the information available in your country.

If you have any further questions, please do not hesitate to contact us at: FWD@ecdc.europa.eu

Thank you very much!

ECDC FWD team
On behalf of EFSA BIOHAZ panel WG on EAEC

*1. Do you have implemented any of the following methods for the detection of EAEC in stool samples?

Multiple choice options are possible
If you have clicked "PCR" or "Other methods" please specify further as indicated in the fields below

- [ ] PCR
- [ ] Bacterial Culture
- [ ] Cell culture
- [ ] Other methods
- [ ] None

If PCR, please specify amplified genes:

2

If "Other methods", please specify which:

2. Do you search diarrhoeal samples for EAEC?

If you did not search for diarrhoeal samples for EAEC please enter "0"

If this information is not known then please enter "unknown"

	Number of samples tested	Number of positive samples
2012		
2013		
2014		

3. Please indicate the number of human EAEC cases in your country in the following years:

If there were no human EAEC cases in the specified year please enter "0"

If this information is not known then please enter "unknown"

	Nr. human EAEC cases
2012	
2013	
2014	

Most common serogroups:

3

4. Please fill in the table below if there is the relevant detailed information available in your country.

Serogroup Number of human EAEC cases/serogroup/year

If this information is not known then please enter "unknown"

	2012	2013	2014
Serogroup 1 *(please specify serogroup)*			
Serogroup 2 *(please specify serogroup)*			
Serogroup 3 *(please specify serogroup)*			
Serogroup 4 *(please specify serogroup)*			

Please specify if there are any other serogroups (number of cases, year)

If this information is not known then please enter "unknown"

5. Please indicate the number of human EAEC outbreaks in your country in the following years:

If there were no human EAEC outbreaks in the specified years please enter "0"

If this information is not known then please enter "unknown"

	Nr. human EAEC outbreaks
2012	
2013	
2014	

5

6. Please fill in the table below if there is the relevant detailed information available in your country.

Serogroup Number of human EAEC outbreaks/serogroup/year

If there were no human EAEC outbreaks in the specified years please enter "0"

If this information is not known then please enter "unknown"

	2012	2013	2014
Serogroup 1 *(please specify serogroup)*			
Serogroup 2 *(please specify serogroup)*			
Serogroup 3 *(please specify serogroup)*			
Serogroup 4 *(please specify serogroup)*			

6

Please specify if there are any other serogroups (number of outbreaks by serogroup, year)

Has a food vehicle been associated with any of the above outbreaks?
(please specify)

7. Are any data on the occurrence of EAEC in humans published in official monitoring reports in your country?

- [ ] Yes
- [ ] No

8. If yes, can you please provide information about such reports, including how they can be accessed?

9. Do you have examples of cases of infection other that gastroenteric in your country over the last 10 years (e.g., UTIs), where EAEC may be involved? If yes, please give brief details.

*Please specify your reporting country and contact point

7

Appendix D – Summary of the replies provided by Member States to the questionnaire on EAEC in cases of human infection

The questionnaire was sent to the 30 countries in the FWD Network.

Number of countries that replied: 22 EU/EEA countries (AU, BE, BG, CZ, CY, DE, DK, EL, ES, FI, FR, HU, IE, IT, LT, LU, LV, NO, RO, SE, SI, UK); response rate 73%.

19/22 (86%) countries have a method available for detection of EAEC (from stool samples). Additional comments from 10 countries: DE, EL, ES, FI, FR, IE, IT, LU, NO, SE):

- PCR method is available but not in routine use;
- *aggR* and *aaic* genes on *E. coli* isolates are detected from bacterial culture of diarrhoeal stools, not in stool samples directly;
- The data is from the National Reference Laboratory but are not representative since EAEC is not under surveillance;
- *aggR* gene is not analysed on all VTEC isolates as it currently is not part of the routine screen. For the years 2012–2014 results are based on a survey for *aggR* on a sample of the VTEC isolates;
- EAEC is not under national laboratory-based surveillance and strains or samples are not collected to the reference laboratory (isolation or serogrouping not done). Results are from one clinical microbiology district hospital laboratory performing a PCR assay for detection of several pathogens, including EAEC, from stool samples from patients with diarrhoea.
- *aggR* is screened for in all *E. coli* isolates received at the Reference Laboratory. However, none of the clinical microbiological laboratories in the country detects EAEC. *aaiC* is screened for in all *eae* negative STEC from patients with severe clinical symptoms (performed at the Reference Laboratory);
- Some labs of primary diagnostics are using *aatA* and/or *aggR* RT-PCR for screening; specialized labs (i.e. reference labs) are using some more gene markers for further typing of isolates (*aap, aaf, pet*);
- Since O104:H4 outbreak in 2011, the National Reference Centre for *E. coli, Shigella* and *Salmonella* implemented a method for the detection of *aggR* gene. This method is only used for the detection of the gene in VTEC strains that are negative for *eae* gene. These data reflect only the number of 'hybrid' strains that have VTEC (*stx* genes) and EAEC (*aggR*) virulence factors;
- Since beginning of 2014, the national reference lab routinely screens all *E. coli* isolates suspected to be verotoxin producers for *aaiC* and *aggR* by PCR.

Methods used for detection EAEC from stool samples (in 19 countries having method for detection of EAEC):

- PCR (17/19; 90%)
- Amplified genes:
  - *aggR* (100%), *aaiC* (67%), *aatA* (33%)
  - additional genes (8 countries reported additional genes): *aafA, aap, amp, aggA, astA, east, eagg, pet, pic, sepA,* new adhesin of enteroaggregative *E. coli* related to the Afa/Dr/AAF family.
- Bacterial culture (9/19; 47%)
  - two countries use this method as their only method, another seven countries use culture together with PCR.

- Other (2/10; 11%)
  - Serotyping and defining the pathovar according to their correlation with common serovars.

Table 3: Number of diarrhoeal samples tested for EAEC/year and number of positive samples (reported by 10 countries) denoted as (minimum number of samples tested in 2012-2014 per country/maximum number of samples tested in 2012-2014 per country): CZ (0/7); DK (Unknown/10,036); IE (182/200); ES (100/169); FI (67/3,058); FR (26/41); HU (1,920/2,804); LU (0/14); RO (23/62); SI (300/700)

Year	2012	2013	2014
Number of samples	13,128	4,290	6,805
Positive samples (%)	994 (7.0%)	129 (2.9%)	290 (4.1%)

CZ: Czech Republic; DK: Denmark; ES:Spain; FI: Finland; IE: Ireland; FR: France; HU: Hungary; LU: Luxembourg; RO: Romania; SI: Slovenia.

Table 4: Number of human EAEC cases/year (reported by 11 countries) denoted as (minimum number of samples tested in 2012-2014 per country/maximum number of samples tested in 2012-2014 per country): BE (0/1); CZ (2/Unknown); DE (372/757); DK (3/5); ES (3/6); FI (10/190); FR (2/3); HU (0/1); IT (0/4); NO (2/7); UK (18/55)

Year	**2012**	**2013**	**2014**
Number of cases	420	832	961

BE: Belgium; CZ: Czech Republic; DE: Germany; DK: Denmark; ES:Spain; FI: Finland; FR: France; HU: Hungary; IT: Italy; NO: Norway; UK: United Kingdom.

Numbers presented in the Tables 3 and 4 are not comparable. Nine countries reported either samples tested/positive samples for EAEC or human cases of EAEC and only three countries reported both.

Table 5: Serogroups/serotypes by year in human EAEC cases (reported by five countries)

Year		
2012	**2013**	**2014**
O111(1)[(a)]	O78 (1) [(a)]	O99:H33
O39:H10	O111 (1) [(a)]	O111ab
O175	O33:H-	
O104:H4 (1)	O181	
	O104:H4 (1) [(a)]	
O99:H33	O63:H-	O25:K2:H4
O111ab	O104	
O111:H12	O128abc:H10	O89:H-
O168	O131	
O128abc:H-	O3	O92:H33
O104		

(a): Number of cases per serogroup/serotype if mentioned by country.

Table 6: Number of EAEC outbreaks by year (reported by two countries)

Year	**2012**	**2013**	**2014**
Number of outbreaks	6	23	24

Table 7: Serotypes reported in the outbreaks (by one country)

Year	**2012**	**2013**	**2014**
Serotype	Unknown	O104 (1) [(a)]	Unknown
	Unknown	O181 (1) [(a)]	Unknown
	Unknown	O131 (1) [(a)]	Unknown

(a): Number of outbreaks per serogroup.

Food vehicles associated with the EAEC outbreaks (two countries reported)

- unpasteurised sheep milk cheese (one country, 2006)
- curry leaves (one country)

Are any data on the occurrence of EAEC in humans published in official monitoring reports in your country/information about such reports:

- No (20 countries)
- Yes, 'Annual epidemiological report on notifiable infectious diseases in Germany; Berlin, Germany; Robert Koch Institute' In chapter the annual numbers of notified infections with EAEC are reported.

Do you have examples of cases of infection other that gastroenteric in your country over the last 10 years (e.g. UTIs), where EAEC may be involved? If yes, please give brief details.

- No (12 countries);
- No, the majority of our positives were from a cohort study indicating that carrier state is 4% and especially related to travellers (one country);

Waterborne infections acquired in Turkey. Many notified EAEC cases (and outbreaks) are travel related (one country).

Appendix E – Relevant data from a number of official EU publications and agencies, reports from the EU RL VTEC and specific reports from MSs utilised in the Opinion

CDC (Centers for Disease prevention and Control), 2011. Estimates of Foodborne Illness in the United States. Available online: http://www.cdc.gov/foodborneburden/PDFs/FACTSHEET_A_FINDINGS_updated4-13.pdf

ECDC (European Centre for Disease Prevention and Control). Fourth external quality assessment scheme for typing of verocytotoxin-producing *E. coli* (VTEC). Stockholm: ECDC; 2014. Available online: http://ecdc.europa.eu/en/publications/Publications/4th-External-Quality-Assessment-typing-of-verocytotoxin-producing-E.-coli-VTEC-web.pdf

ECDC (European Centre for Disease Prevention and Control). Fifth external quality assessment scheme for typing of verocytotoxin-producing *Escherichia coli* (VTEC). Stockholm: ECDC; 2014. Available online: http://ecdc.europa.eu/en/publications/Publications/VTEC-EQA-2014.pdf)

EFSA (European Food Safety Authority), 2011. Shiga toxin-producing *E. coli* (STEC) O104:H4 2011 outbreaks in Europe: Taking Stock. EFSA Journal 2011;9(10):2390, 22 pp. doi:10.2903/j.efsa.2011.2390

EFSA (European Food Safety Authority), 2013. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011. EFSA Journal 2013;11(4):3129, 250 pp. doi:10.2903/j.efsa.2013.3129

EFSA (European Food Safety Authority), 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA Journal 2015;13(1):3991, 165 pp. doi:10.2903/j.efsa.2015.3991

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2011. Scientific Opinion on the risk posed by Shiga toxin-producing *Escherichia coli* (STEC) and other pathogenic bacteria in seeds and sprouted seeds EFSA Journal 2011;9(11):2424, 101 pp. doi:10.2903/j.efsa.2011.2424

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards (BIOHAZ), 2013. Scientific Opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. EFSA Journal 2013;11(4):3138, 106 pp. doi:10.2903/j.efsa.2013.3138

EU RL VTEC (European Union Reference Laboratory VTEC), 2012. Report of the 10th inter-laboratory study (PT10) on Verocytotoxin-producing *E. coli* (VTEC) identification and typing – 2012-2013. Available online: http://www.iss.it/binary/vtec/cont/PT10_Report.pdf

EU RL VTEC (European Union Reference Laboratory VTEC), 2013a. Detection of enteroaggregative *Escherichia coli* in food by Real Time PCR amplification of the aggR and aaiC genes. Rome, Italy. Available online: http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_05_Rev_1.pdf

EU RL VTEC (European Union Reference Laboratory VTEC), 2013b. Report of the 11th inter-laboratory study (PT11) on the identification and typing of Verocytotoxin-producing *E. coli* (VTEC) and other pathogenic *E. coli* strains - 2013. Available online: http://www.iss.it/binary/vtec/cont/Report_PT11_5.pdf

EU RL VTEC (European Union Reference Laboratory VTEC), 2014. Report of the 13th inter-laboratory study (PT13) on the identification and typing of Verocytotoxin-producing *E. coli* (VTEC) and other pathogenic *E. coli* strains - 2014. Available online: http://www.iss.it/binary/vtec/cont/Report_PT13.pdf

FSA (Food Standards Agency), 2000. A report of the study of infectious intestinal disease in England. London: The Stationery Office. Available online: http://www.esds.ac.uk/doc/4092%5Cmrdoc%5Cpdf%5C4092userguide6.pdf

PHE (Public Health England), 2013. Outbreak of Salmonella agona phage type 40 associated with the street spice festival, Newcastle upon Tyne February / March 2013. ILOG 8168. Available online: http://www.newcastle.gov.uk/sites/drupalncc.newcastle.gov.uk/files/wwwfileroot/environment/environmental_health/20130617_street_spice_oct_report_-_final.pdf

WHO (World Health Organization), 2012. Critically Important Antimicrobials for Human Medicine, 3rd revision. World Health Organization 2012, ISBN: 978 92 4 150448 5

Appendix F – What does multi-locus sequence typing (MLST) and whole genome sequencing (WGS) tell us about EAEC?

Mosaic genomic structure of E. coli

The *Escherichia coli* genome structure comprises the ancestral backbone genes that define *E. coli* and a large number of relatively recently introduced genes in the remainder of the chromosome. The ancestral backbone genes have undergone a slow accumulation of vertically acquired sequence changes or mutations. In contrast, the recently introduced genes have been acquired *via* numerous, independent horizontal gene-transfer events at many discrete sites across the genome, with certain loci serving as universal insertion targets used independently in separate lineages.

The net result is a *mosaic genome structure* in which newly acquired genes are placed into a framework of approximately 3,000 genes common to *E. coli* – the conserved core genome (Welch et al., 2002). This mosaic structure provides a framework for commensal and low virulence strains to act as genetic repositories for virulence factors and may increase their pathogenic potential.

High level of genetic diversity observed in strains of EAEC

Previous studies have shown that EAEC are a heterogeneous group of pathogens with respect to both phenotypic and genotypic characteristics (Jenkins et al., 2006a; Okeke et al., 2010). WGS has provided further evidence of this high level of genetic diversity (Rasko et al., 2008; Dallman et al., 2014). This diversity is apparent at every level, from the overall phylogeny to the genomic architecture of the pAA plasmid and the presence and absence of putative virulence genes (Jenkins et al., 2005; Dallman et al., 2014).

Rasko et al. (2008) analysed two EAEC genomes and concluded that there were a significant number of truly unique genes (TUG) in each genome – 308 in EAEC O42 and 155 in EAEC 1010-1 – and few pathovar specific genes. They highlighted that EAEC included atypical strains or 'outliers' that further confounded the genomic dataset associated with this pathogroup.

EAEC lineages have evolved many times

Multi-locus sequence typing (MLST) and whole genome sequencing (WGS) data provide evidence that prevailing 'successful' lineages have evolved independently many times as they are spread throughout the entire *E. coli* population (Dallman et al., 2014). Pupo et al. (2000) suggested that strains of *E. coli* act as genetic repositories with the ability to acquire DNA from multiple sources as well as the ability to act as donors. Acquisition of the appropriate pathogenic features may result in transfer of the ability to cause disease to a commensal or low level pathogen isolate. Conversely, pathogens may be able to revert to a commensal state by loss or donation of DNA.

The successful lineages, as defined by MLST complex, appear to be globally distributed. There is some evidence that certain lineages may be more pathogenic than others (Chattaway et al., 2014b).

Multiple events have led to the independent evolution of EAEC

Clonal Frame analysis showed that EAEC mutation and recombination rates vary across the lineages and that both events play an important part in the evolution of EAEC. Although the dataset was limited, Chattaway et al. (2014b) showed that recombination rate was higher in the STs associated with disease. WGS data suggests that prophage and phage elements play a significant role in the evolution of certain *E. coli* pathovars (Rasko et al., 2008).

Appendix G – Protocol developed by the EU RL for *E. coli* for the detection of EAEC in food by Real Time PCR amplification of the *aggR* and *aaiC* genes

EU Reference Laboratory for *E. coli*
Department of Veterinary Public Health and Food Safety
Unit of Foodborne Zoonoses
Istituto Superiore di Sanità

Detection of Enteroaggregative *Escherichia coli* in food by Real Time PCR amplification of the *aggR* and *aaiC* genes

1. Aim and field of application

The large outbreak occurred in Germany during Summer 2011 associated to the consumption of sprouts was caused by a VTEC strain which possessed atypical characteristics, and did not fit the current definition of pathogenic VTEC. In fact, the epidemic strain did not possess the *eae* gene, encoding the adherence factor intimin, but possessed genes that are typical of the Enteroaggregative *Escherichia coli* (EAggEC) group. The strain was indeed an EAggEC that had acquired the *stx2a*-converting bacteriophage. This matter has highlighted the importance to screen foodstuffs for EAggEC too, which may represent an emerging public health concern.

The present procedure describes a molecular methodology to screen food samples for the presence of EAggEC by the detection of targets designed on the *aggR* and *aaiC* genes, which represent genetic markers characteristic for this group of pathogenic *E. coli*. The same genetic markers have been indicated by the European Centre for Disease Prevention and Control (ECDC) and by the European Food Safety Authority (EFSA) for the identification of EAggEC strains.

2. Screening of food samples

Enrichment cultures are obtained by adding 25 g test portions of the food specimen (or 25 ml of liquid) to 225 ml of Buffered Peptone Water (BPW), homogenizing in a peristaltic blender, and incubating at 37 ± 1 °C for 18-24 h.

One ml of the enrichment culture is used for DNA extraction. This step is accomplished by any method in use in the laboratory for the extraction and purification of DNA from food enrichment cultures.

The Real Time PCR targeting the *aggR* and *aaiC* genes is performed with the primers and probes reported in Annex 1. Amplification conditions will depend on the system used and will refer to the instructions supplied with the instrument and the kit of choice, and should be set up in each laboratory. However, standard reaction conditions together with a two-steps thermal profile applied at EU-RL VTEC are included in Annex 1.

Enrichment cultures positive for the presence of *aggR*, or *aaiC*, or both genes are streaked onto suitable solid media (MacConkey agar plates or other media suitable for *E. coli* isolation, such as TBX) for attempting the isolation. This step is accomplished as follows:

- ❑ Pick up to 50 colonies with *E. coli* morphology.
- ❑ Point-inoculate on Nutrient Agar (NA) (single colonies).
- ❑ Test the isolated colonies or pools of 10 colonies by real time PCR for the presence of the gene(s) detected in the screening step.
- ❑ Subculture the positive colony for further characterisation.

3. Real Time PCR amplification of the *aggR* and *aaiC* genes

The present annex illustrates the primers and probes sequences and the Real Time PCR conditions for the amplification of two genetic markers of typical EAggEC: the plasmid-located gene *aggR*, coding for a transcription regulator, and the chromosomal gene *aaiC*, which is part of the *aai* gene cluster, encoding a type VI secretion system.
The protocol is based on the 5'-nuclease PCR assay. The primers and probes targeting the *aggR* and *aaic* genes have been deployed and tested at the EU-RL VTEC and their sequences are reported in the table below.
The nature of the Reporter and Quencher is not indicated, as it may depend on the Real Time PCR apparatus available in the laboratory.
The amplification of the two target genes can be performed simultaneously in a triplex Real Time PCR, including the assays for the two genes and the internal amplification

control (IAC), if the Real Time Apparatus allows the simultaneous detection of three different fluorophores.

Table. DNA sequence and characteristics of the primers and probes used for the detection of EAggEC.

Target gene	Primer/Probe name	Forward Primer, Reverse Primer and Probe sequences (5'-3')	Amplicon size (bp)	Location within sequence	Sequence accession number
aggR	*aggR* FWD	GAATCGTCAGCATCAGCTACA	102	47738-47718	CU928159.2
	aggR REV	CCTAAAGGATGCCCTGATGA		47637-47656	
	aggR probe	CGGACAACTGCAAGCATCTA		47697-47678	
aaiC	*aaiC* FWD	CATTTCACGCTTTTTCAGGAAT	160	3385498-3385477	NC_011748.1
	aaiC REV	CCTGATTTAGTTGATTCCCTACG		3385339 - 3385361	
	aaiC probe	CACATACAAGACCTTCTGGAGAA		3385427-3385405	

Real Time PCR conditions:

Master Mix 2X	to 1X (usually containing $MgCl_2$ to final concentration of 3mM)
Primer Fwd	500 nM
Primer Rev	500 nM
Probe	200nM
DNA	X (2 µl of DNA purified from 1 ml of culture can be sufficient)
Water	to final volume

The primers and probes have been evaluated at the EU-RL VTEC with a Corbett Rotorgene, by using the following basic two steps thermal profile:

95 °C 10 minutes

35-40 cycles of:

- 95 °C 15 seconds
- 58 °C 60 seconds

4. References

- Chaudhuri RR, Sebaihia M, Hobman JL, Webber MA, Leyton DL, Goldberg MD, Cunningham AF, Scott-Tucker A, Ferguson PR, Thomas CM, Frankel G, Tang CM, Dudley EG, Roberts IS, Rasko DA, Pallen MJ, Parkhill J, Nataro JP, Thomson NR, Henderson IR.
- Complete genome sequence and comparative metabolic profiling of the prototypical enteroaggregative Escherichia coli strain 042. PLoS One. 2010;5:e8801.
- EFSA. Scientific Opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. EFSA Journal 2013;11(4):3138.
- Nataro JP. Enteroaggregative *Escherichia coli* pathogenesis. Curr Opin Gastroenterol. 2005;21:4-8.
- Scavia G, Staffolani M, Fisichella S, Striano G, Colletta S, Ferri G, Escher M, Minelli F, Caprioli A. Enteroaggregative Escherichia coli associated with a foodborne outbreak of gastroenteritis. J Med Microbiol. 2008;57:1141-6.