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Elucidating the Epidemiology of Human Salmonellosis: The Value of Systematic Laboratory Characterisation of Isolates

P. McKeown[1], P. Garvey[1] and M. Cormican[2]
[1]Health Protection Surveillance Centre, Dublin
[2]National Salmonella Reference Laboratory, Bacteriology Department
Galway University Hospital
Ireland

1. Introduction

Infection with non-typhoidal *Salmonella enterica* (NTS) represents a significant burden of gastroenteric illness upon the world's population. Most enteric *Salmonella* infection is zoonotic, transmitted from healthy vertebrate animals to humans, largely by means of contaminated food. The reported incidence of enteric salmonellosis increased rapidly after the Second World War in association with progressive industrialisation of the food supply, at a time when the incidence of typhoid was declining, consequent upon the extensive development of water treatment and waste disposal systems, coupled with the pasteurization of milk.[1] As a result, infection with NTS displaced typhoid in the developed world as the major threat to human health from *Salmonella* during the 20th century.

Salmonellae have evolved into a diverse genus of *Enterobacteriaceae*; some members being adapted to specific hosts with others having a broad host range. In addition to their wide spectrum of zoonotic hosts, salmonellae vary greatly in age (*S.* Typhi having emerged more recently than *S.* Typhimurium), in lineage and in clonality. Accordingly, a variety of genome-based methods must be used in order to provide appropriate methods for characterisation of different variants

One hundred million cases of salmonellosis are estimated to occur globally each year. Estimates of incidence range from 32 cases/100,000 population in high income areas of the Asia Pacific region to 3,600/100,000 population in Southeast Asia.[2] Annually, this results in 155,000 deaths worldwide. Mortality rates from salmonellosis are highest in East and Southeast Asia and lowest in the developed countries of Europe, North America and Oceania.

About 80% of all salmonellosis cases are estimated to be foodborne (rising to 94% in the United States).[3] Reported incidence varies widely, not least in developed countries, reflecting both real differences in incidence (driven by variations in farming/food production practices, the existence of *Salmonella* Control Programs and food consumption patterns), and the effects of variability in surveillance parameters, and health care and

diagnostic systems. In Western Europe and in high-income regions of North America, total incidence (which includes confirmed NTS cases combined with projections based upon population models) is estimated to be 220/100,000 and 495/100,000 respectively.[2]

These figures do not, however, paint the full picture. Although there is marked regional variation, there has been a steady decrease in the total confirmed notification rates for salmonellosis in the European Union over the last six years from 196,000 cases in 2004 to 108,000 cases in 2009 (or 21.6 cases /100 000 population), representing an average 12% fall per year.[4] The incidence has remained static in Ireland (at 10 cases/100,000 between 2006 and 2008) but has fallen in the UK (from 23 to 19/100,000 cases) over the same period. Certain countries, however, have seen marked increases in reported incidence between 2006 and 2008 (from from 31 to 67 cases/100,000 in Denmark and from 16 to 39 cases/100,000 in Malta) while others report steep declines in incidence (such as the Czech Republic falling from 236 to 103 cases/100,000 and from 64 to 52 cases/100,000 in Germany).[5]

In the United States, approximately 40,000 laboratory-confirmed cases of *Salmonella* infection are reported annually to the National *Salmonella* Surveillance System in the United States, giving an annualised incidence rate, in 2006 of 13.3 cases per 100,000 population (CDC, 2011).[6]

Under-ascertainment of enteric salmonellosis is a significant concern. In the UK, the ratio of *Salmonella* isolates reported nationally to cases occurring in the community has been estimated as being 4.7, i.e. 3.7 undetected community cases for each laboratory confirmed case included in national statistics.[7]

Salmonellosis underascertainment has been estimated in a range of European countries using an intriguing method by Swedish researchers.[8] Investigators calculated the incidence of salmonellosis acquired overseas among returning Swedish travellers on a country-specific basis and compared this derived incidence against nationally reported incidence in the country in which the case had acquired their infection. As a result, they estimated that there was significant variation in the ratio of underdetection by the national reporting systems of the countries involved, ranging from less than one in the case of Finnish and Icelandic systems (i.e. these systems were more sensitive at detecting salmonellosis than the Swedish travel-based system) to 98 and 270 in the case of Greek and Bulgarian systems, suggesting that these systems were considerably less sensitive at detecting salmonellosis than the Swedish travel-based system. Interestingly, the underdetection index for Ireland was 4.3 - precisely the same as that found for the UK.[8] The authors note that the behaviours and risks of Swedish travellers may not be fully representative for those of the native population; nevertheless, it provides an interesting comparative snapshot of potential *Salmonella* underascertainment in Europe.

The Centers for Disease Control and Prevention (CDC) has recently estimated that the true annual incidence of salmonellosis in the US to be 1,027,561 non travel-associated domestic cases,[3] highlighting the perennial issue of infectious intestinal disease underascertainment. Using CDC's estimates, it can be calculated that for every laboratory confirmed case of domestically acquired salmonellosis, there are approximately 25 clinical cases that are not laboratory confirmed.

Salmonellae are effective outbreak organisms and extensive outbreaks of salmonella occur frequently, ranging in size from a couple of cases, to tens of thousands of cases. A significant number of these outbreaks are international in distribution and have involved a wide range of food products including chocolate,[9,10,11] imported eggs,[12] infant formula,[13] fresh basil,[14] raw milk cheese,[15] pork,[16] rucola lettuce,[17] sprouts,[18] pre-cooked meat products,[19] lasagne,[20] pet products,[21] sesame seeds,[22] raw almonds,[23] peanuts,[24] peanut butter,[25] and ready-to-eat vegetables.[26] In addition, in 2008, the European Food Safety Authority reported 490 confirmed foodborne outbreaks of salmonellosis resulting in 7,724 cases, 1,363 hospitalisations and 118 deaths.[27]

In considering the relative and absolute burden of human salmonellosis based on data from the developed world, it is perhaps striking that NTS infection remains a potent public health and clinical challenge, although the majority of developed nations have both well-developed surveillance systems to detect human salmonellosis (and the outbreaks that result), and farm-based and food hygiene surveillance systems specifically designed to control food-borne NTS infection. There is however, some comfort in the static or falling incidence of salmonellosis in many developed countries.

A range of emerging factors facilitate the rapid distribution of all foodborne microbes, including *Salmonella:* globalization of the food supply, an aging and highly mobile population able to distribute an increasingly diversified intestinal flora more widely, a growing proportion of the population at special risk due to immunosuppressive diseases such as cancer, or consuming pharmaceutical agents that inhibit either the immune system (such as cytotoxic agents) or protective gastric acid secretion (such as proton pump inhibitors), changing dietary preferences for raw or lightly cooked food, intensification in farming practice, environmental encroachment with greater exposure to novel pathogens, climate change and international travel and trade between countries.[28]

This importance of increased movement of populations and food is partly reflected in the growing proportion of NTS infection attributed either to international travel or to the consumption of imported food. Up to half of Irish *Salmonella* infections are reported as being acquired outside Ireland.[29] More than 60% of cases of human salmonellosis in Denmark in 2007 were associated with consumption of imported meat or with international travel.[30] The Smittskyddsinstitutet, the Swedish government agency with responsibility to monitor the epidemiology of communicable diseases, estimates that more that 74% of reported NTS infections identified in Sweden are acquired on trips outside that country.[31]

The incidence of salmonellosis increased markedly during the 1970s and 1980s. Between 1976 and 1986, reported infections due to *S.* Enteritidis (a commensal primarily of poultry, particularly chickens) increased more than six-fold in the north-eastern United States,[32] while the incidence of infections due to *S.* Typhimurium remained static.[33] This led investigators to wonder if they were witnessing the onset of a novel pandemic.[34] A number of theories as to the underlying explanation of this increase were considered, including clonal expansion of a single, more virulent variant of S. Enteritidis. It was concluded, however, that this upsurge was most likely triggered by *S.* Enteritidis occupying the ecological niche left vacant by the established avian *Salmonella* pathogens, *S.* Pullorum and S. Gallinarum, when those subtypes had been largely eliminated from poultry flocks,[34] with transmission of human disease being amplified by the progressive intensification of poultry farming.

2. Identification and linking of cases

In Ireland, as is common in most other developed countries, the appearance of a clinical case of salmonellosis will prompt a number of public health and microbiological responses. The management of the individual patient may not require either detailed characterisation or antimicrobial susceptibility testing since *Salmonella* gastroenteritis is generally self-limiting. From a public health perspective however, detailed characterisation of the isolate may help to determine the extent of linkages, and potential sources. Preliminary interviewing of the case seeks to determine if there is epidemiological evidence of linkage (to other cases or a possible source) and to determine if the case is in a high risk category (in this case, high risk means that they are at increased risk of spread of the *Salmonella* strain; for example, if the case were a food handler and confirmed as having salmonellosis s/he would pose a risk of onward transmission). If there is laboratory evidence of linkage, each potentially linked case is administered an extensive national *Salmonella* Trawling Questionnaire, designed to question the case in close detail to determine if there are exposures common to other, similar cases.[35]

It is the knitting together of in-depth clinical public health interviews and definitive characterisation of isolates from clinical (and frequently food and animal) specimens, that facilitate the identification of common sources of infection, therefore close collaboration between public health microbiologists and epidemiologists is essential to effective prevention and control.

2.1 Microbiological identification

Almost all human cases of NTS infection are associated with a single species; *Salmonella enterica.* However, the highly developed system of sub-classification within the species is valuable in linking isolates from different human and non-human sources.

Confirmation of the diagnosis of human salmonellosis and further characterisation of the isolate entails, initially, the bacteriological isolation of the organism from a clinical specimen. Clinical samples are typically stool specimens but blood, urine, spinal fluid, joint fluid, pus and tissues may be examined. The isolation of *Salmonella* from faeces requires the use of media that allows for the preferential growth of *Salmonella* from among the complex mixture of bacteria that comprise the normal gastrointestinal flora. This is achieved by direct culture on selective agar media such as Xylose-Lysine-Desoxycholate agar (XLD) or chromogenic agars. To enhance detection of low numbers of *Salmonella*, stool samples are also, generally inoculated into a selective enrichment broth (often Selenite F broth), which is plated to selective media after overnight incubation. This two-step process means that, although a preliminary indication that a culture is negative on primary plating is typically available at 24 hours, a definitive "Not Detected" report is typically not available for 48 hours. Specimens from normally sterile body sites are typically cultured on non-selective agar media (for example blood agar) or broth because there is no requirement to suppress competing normal flora. Urine samples are a special case because many clinical laboratories do not characterise all significant urine isolates beyond the level of *Enterobacteriaceae* (coliforms). As a result, *Salmonella* urinary tract infections may go unrecognised.

It is important that the limitations of the methods used for detection are understood by practitioners. The reliability of the result is critically dependent upon the quality

management systems in place in the clinical laboratory and, ideally, such laboratories should be accredited to the ISO-15189 standard. Even with rigorous control of quality, microbiologists should report samples as "*Salmonella* not detected" (or words of similar meaning), avoiding such terms as "*Salmonella* negative" or "*Salmonella* absent". For epidemiologists and food safety agencies it is important to understand that even if a laboratory uses the term "negative" or "absent" in informal communication, failure to detect *Salmonella* on culture does not entirely exclude the possibility of infection.

Provisional positive results may be available within 24 hours (from the primary plate) or within 48 hours if cultured only from subculture of enrichment broth. Definitive confirmation of the isolate and antimicrobial susceptibility testing may require an additional working day although a provisional positive report from a laboratory with skilled scientists and effective quality systems generally has a very high degree of reliability. Confirmation of a suspect colony as being due to *Salmonella* may be achieved by biochemical and serological characterisation or by molecular methods (the latter may allow for more rapid confirmation).

The extent to which clinical laboratories characterise isolates in their own laboratory before submission to a reference laboratory, and the frequency with which isolates are submitted to reference laboratories, may depend on experience, skills sets, resources and funding/reimbursement systems, and ease of access to reference laboratory services. Although antimicrobial agents are not required in most patients with *Salmonella* gastroenteritis, this can represent useful preliminary characterisation and is essential to guide therapy in those with invasive disease. Antimicrobial susceptibility testing should be performed by standardized methods [European Committee on Antimicrobial Susceptibility Testing EUCAST), or Clinical Laboratory Standards Institute (CLSI) I or International Standards Organization (ISO 20776-1) or by commercial systems validated against these standards. Measurements (diameter of zone of inhibition or minimum inhibitory concentration; MIC) should be interpreted with reference to EUCAST or CLSI interpretive criteria. The use of non-standardised methods for performance or interpretation does not form a sound basis for clinical or public health decision-making. The use of national standards may provide effective clinical guidance but may limit comparability of data with other countries.

Antimicrobial resistance patterns can provide useful supplementary information about the degree of relatedness of members within a particular serotype. Phage typing of serotypes such as *S.* Typhimurium, *S.* Enteritidis and *S.* Agona has been used extensively for epidemiological purposes. Phage typing is a rapid and discriminatory phenotypic method. Interpretation is somewhat subjective; standardization is difficult and phages are not generally available from commercial sources.[36] However, external quality assessment programmes in Europe have confirmed, with a common stock of phage (provided through HPA Colindale) coupled with, common methods and training, that national reference laboratories can produce comparable phage-typing results for *S.* Enteritidis and *S.* Typhimurium. In the past, plasmid profiling was used extensively in identifying outbreak strains and may still be useful in certain settings.

Further typing and subtyping by genome-based methods including pulsed field gel electrophoresis (PFGE),[37] multiple locus variable number tandem repeat (VNTR) analysis

(MLVA), multilocus sequence typing (MLST) can add value, however the discriminatory power of each molecular method may vary based on the serotype under consideration. In the not-too-distant future, single nucleotide polymorphisms (SNPs) and indeed whole genome sequencing may be employed to aid in investigating certain outbreaks.[38]

2.2 Case linkage

Linking of cases of salmonellosis (a necessary first step in the identification of outbreaks) has, by convention, been undertaken using the traditional epidemiological process of describing cases in terms of time, place and person whilst looking for potential linkages between cases that might give a clue as to a possible common source for infection.[39,40] At an early stage, this epidemiological information should be combined with information on characterisation of the isolates, as a first step in determining which cases should be included (and excluded) as being considered part of a particular cluster or outbreak. Serotype and antimicrobial-resistance patterns are generally available at an early stage and may provide pointers that isolates might belong to a homogenous group supporting the possibility of a common source.

In countries with smaller populations and/or low reported incidence of infection, the appearance of a cluster of isolates of an unusual serotype may be readily detected and prompt an investigative response. Countries with larger populations and higher incidence may have greater difficulty in identifying a cluster among the background levels and may have a higher threshold for response. Advanced systems of triggering exist in some countries, and are based on mathematical models to produce an automated alert once an expected threshold is exceeded.

Serotyping and antimicrobial-resistance patterns are of limited value however, in relation to serotypes that are very common and widely distributed. In Ireland, in 2008, the five commonest *Salmonella* serotypes (*S.* Typhimurium, *S.* Enteritidis, *S.* Agona, *S.* Virchow and *S.* Java) accounted for 70% of all isolates (see Figure 1).[41] Isolation of such a common serotype from two sources (i.e. from two cases or from a case and a food item) may well be a chance finding and does not represent persuasive evidence of an epidemiological link. Furthermore, isolates of *S.* Enteritidis are often susceptible to all or most antimicrobial agents tested routinely so that most reference laboratories receive a large number of fully susceptible *S.* Enteritidis isolates.. However, thisdegree of identification will not be adequate to support public health decision making regarding the degree of relatedness of strains and hence the extent of linking that might exist between isolates. It is in this situation that the molecular typing methods briefly outlined above add most value.

There are a number of key principles that must be considered in interpreting laboratory data. First, the extent of characterisation performed should be appropriate to address the epidemiological and public health issues of concern. Serotyping may be sufficient in some cases (especially for rare serotypes) but may be quite inadequate in others. Second, data generated by laboratory typing must always be interpreted in the context of: (1) the current epidemiological situation, (2) decades of accumulated published experience about routes of transmission and (3) an understanding of limitations of the methods used. It is rarely, if ever, appropriate to make a determination that isolates are linked or unlinked based solely on laboratory typing data. It is important to remember that regardless of the sophistication

of the typing methods used, the best that can be achieved is the demonstration of evidence of a link between isolates. It is not possible, based on typing methods alone, to determine the pathway of transmission (and hence to establish causality), that is to say typing does not allow one to determine if the person was infected from the food or if the infected person contaminated the food.

Epidemiologists should be aware of the potential for pseudo-infection and pseudo-outbreaks related to laboratory cross-contamination of samples. This can be a particular issue when a laboratory external quality assessment/proficiency programme has recently included a *Salmonella* isolate in a round of testing. It may be helpful to clarify if the isolate was detected on primary agar plate culture, or only following enrichment. In our experience, growth of multiple colonies of *Salmonella* on the primary agar plate is unlikely to be due to laboratory cross contamination. However, when there is no growth on the primary agar plate but *Salmonella* is isolated from the Selenite F broth, it is important to consider the possibility of cross contamination, in particular if the laboratory has cultured a similar isolate from a clinical sample or external quality assessment sample in the previous few days.

3. Irish data on *Salmonella* isolates

Data from Irish national sources give a clear illustration of the degree of variability among and within *Salmonella* serotypes. In Ireland, all *Salmonella* isolates received at the National *Salmonella*, *Shigella* and *Listeria* Reference Laboratory (NSSLRL) at Galway (this includes all human clinical, and a number of veterinary and environmental isolates) are serotyped, susceptibility to a suite of antimicrobial agents is assessed and all isolates of *S.* Typhimurium and *S.* Enteritis are differentiated by phage typing. Since 2009, MLVA has also been applied routinely to *S.* Typhimurium isolates providing an additional level of discrimination. Additional molecular methods such PFGE are applied selectively during cluster/outbreak investigations.

In all, about 175 different *Salmonella* serotypes were reported to the NSSLRL between 2000 and 2010 among Irish clinical isolates. However, the current epidemiology of *Salmonella* in Ireland is dominated by two serotypes, *S.* Enteritidis and *S.* Typhimurium (including monophasic Typhimurium). These two serotypes accounted for 20% and 38% respectively, of human clinical isolates identified in Ireland in 2010, while other serotypes made up the remaining 42% of isolates (Figure 1). This represents a change in the relative importance of these serotypes since earlier in the decade when *S.* Enteritidis was consistently the most common serotype among Irish clinical isolates.

Within *S.* Typhimurium, approximately 90 definitive types have been detected since 2000, the 10 most common of which are depicted in Table 1. Overall, DT104 and DT104b have been the most common phage types detected. Antimicrobial susceptibility patterns and molecular typing (MLVA and PFGE) indicate significant diversity within these phage types.

Within *S.* Enteritidis, although PT4 and PT1 have been the most common phage types since 2000, the number and proportion of both have declined markedly in recent years; in 2010, PT14b was the most common type (Table 2). MLVA provides increased discrimination within common *S.* Enteritidis phage types; however, unlike S. Typhimurium, there is not at present a clear consensus on a standardized approach to MLVA for this serotype.
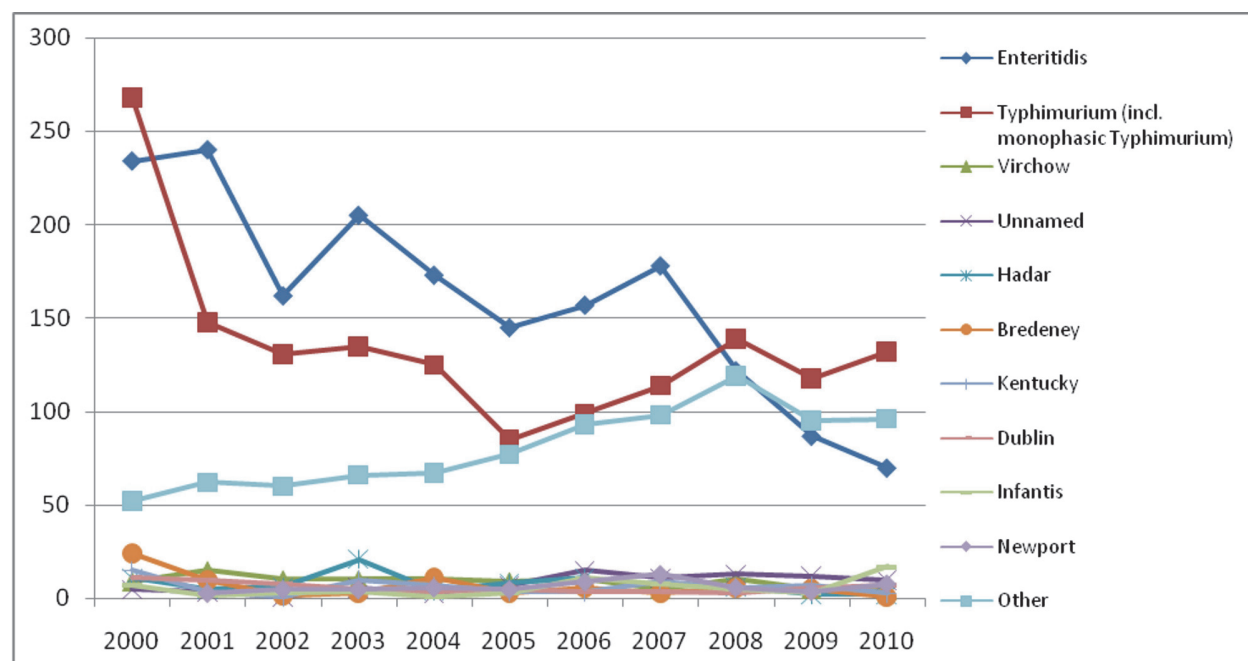
[Data source: NSSLRL, Unpublished data]

Fig. 1. Annual number clinical Salmonella isolates by serotype, Ireland 2000-2010 – [Top ten individually represented with all others combined]

Phage type	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	Total (%)
DT104	194	39	25	22	48	37	24	21	28	24	26	**488 (33%)**
DT104b	23	48	49	67	23	13	29	14	27	14	14	**321 (21%)**
DT193	11	11	16	4	2	5	11	13	18	27	18	**136 (9%)**
U302	8	6	9	8	2	1	7	4	9	3	1	**58 (4%)**
Untypable	1	2	0	1	0	7	0	9	7	10	15	**52 (3%)**
DT120	1	6	0	0	3	0	1	19	6	6	6	**48 (3%)**
DT8	1				1			1		5	28	**36 (2%)**
DT12	4	1	6	8	1	3		2	4			**29 (2%)**
U311	0	3	3	3	1	0	0	0	0	6	6	**22 (1%)**
Other	25	32	23	22	44	19	27	31	40	23	18	**304 (20%)**
Total	268	148	131	135	125	85	99	114	139	118	132	**1494 (100%)**

[Data source: NSSLRL, Unpublished data]

Table 1. Annual Number S Typhimurium by Definitive Type, Ireland 2000-2010 [Top nine individually represented with all others combined]

Phage type	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	Total
PT4	162	86	36	58	43	19	33	70	22	7	9	**545 (31%)**
PT1	26	74	51	53	48	44	29	13	23	9	14	**384 (22%)**
PT21	5	10	5	21	18	12	26	13	22	11	6	**149 (8%)**
PT14b	8	6	8	7	11	22	19	17	11	20	17	**146 (8%)**
PT8	4	7	12	10	10	20	17	35	14	13	4	**146 (8%)**
PT6	3	3	16	13	10	3	4	12	8	4	2	**78 (4%)**
PT6a	8	12	9	11	11	3	1	2	1	4	1	**63 (4%)**
PT13a		6	3	5	1	1	1	1	2	2	1	**23 (1%)**
Untypable	2	1	2	1		2	2	3	1	1	5	**20 (1%)**
Other	16	35	20	26	21	19	25	12	18	16	11	**219 (12%)**
Total	**234**	**240**	**162**	**205**	**173**	**145**	**157**	**178**	**122**	**87**	**70**	**1773 (100%)**

[Data source: NSSLRL, Unpublished data]

Table 2. Annual Number S Enteritidis by Phage Type, Ireland 2000-2010 [Top nine individually represented with all others combined]

4. Outbreaks

Salmonellae are relatively hardy microorganisms, surviving prolonged periods in frozen storage,[42] and in manure and manure-soil mixtures;[43] food at room temperature or slightly above, provides very favourable conditions for their multiplication. A relatively small inoculum (<1000 cells) is generally sufficient to produce clinical illness or colonisation.[44] Many serotypes of NTS have a particularly broad host range and may persist in the gastrointestinal tract of animal hosts for extended periods. These characteristics, coupled with the steady globalisation of the human food supply and global travel, contribute to the potential of *Salmonella* to cause both well-demarcated local and global outbreaks as well as periodic emergence of clonal groups which disseminate in a more diffuse manner (for example, as monophasic *Salmonella* Typhimurium has done in recent years).

Salmonellae spread readily by means of food, from zoonotic hosts and directly from person to person. The progressive intensification and mechanisation of production, and globalisation of distribution of our food supply, has meant that outbreaks of *Salmonella* can be very extensive, and their sources, deeply embedded. In the United States during 2008-9, a multistate outbreak of *Salmonella* Typhimurium - linked to peanut butter – resulted in more than 700 cases of illness.[45] Its final cost was expected to exceed $1Bn.[46] In 2008, an outbreak of *Salmonella* Agona associated with a food production facility in Ireland led to the recognition of 163 associated cases of illness across Europe including two deaths; the implicated facility exported 800 tonnes of cooked food product across the world each week

(for a fuller description of this outbreak, see below).[19] An important facet of *Salmonella* outbreaks (in common with many other outbreak pathogens) is that the number of cases detected by investigation almost invariably represents a significant underestimate of the true burden of illness resulting from a particular source. It is also important to note that although enteric salmonellosis is a self-limiting illness in most people, in most substantial outbreaks, a number of associated deaths (particularly among the vulnerable and elderly) is not uncommon.

Outbreaks of salmonellosis are frequent events in developed countries, but show a definite decrease in the EU from 2,201 outbreaks in 2007 to 1,722 in 2009.[47]

5. Examples where molecular microbiology was influential in hypothesis generation or source implication during outbreak investigations in Ireland

The consistent and standardised application of *Salmonella* typing methods has enabled a detailed understanding of the baseline or expected incidence of specific *Salmonella* subtypes in Ireland (as is the case in almost all developed countries). This has been of critical importance in the detection of potential clusters based on deviation from the expected incidence. Close collaboration between epidemiologists and microbiologists is essential in forming a judgement as to which clusters are appropriate for epidemiological investigation. Many of the laboratory techniques are applied in reference laboratories across the developed world using standardised protocols. Communication of laboratory results (including results of genotyping studies) in standardised formats through channels such as those of the European Centre for Disease Prevention and Control (ECDC) and bilaterally between National Reference Laboratories and National Epidemiological Institutes can be vital in both detection and management of international outbreaks.

A large outbreak of *Salmonella* Agona originating in Ireland, involving a number of European countries and linked to an Irish Food manufacturer in 2008 neatly illustrates the concept of hypothesis generation.[19] In this outbreak, six cases of *Salmonella* Agona, each having the same unique PFGE profile (SAGOXB.0066) were identified within a two week period (prior to this outbreak, six cases would be a typical annual total for *Salmonella* Agona isolates in Ireland). Within two weeks of the first cases having been identified, a review of *Salmonella* Trawling Questionnaires, coupled with emerging microbiological evidence of the outbreak strain (displaying the PFGE profile of the clinical isolates) being identified on the premises of an Irish food manufacturer, and in food outlets supplied by this same company, led investigators to hypothesise that a number of food items produced by the Irish Food manufacturer were the vehicles of infection via these food outlets. From data provided through the *Salmonella* Trawling Questionnaires, three quarters of cases reported consuming food from take-away chains and eating sandwiches containing chicken or pork/ham.

Together, epidemiological and microbiological evidence augmented one another in this outbreak. The epidemiological evidence pointed to the commonality of exposure to particular types of retail food outlets (take away chains), to particular food types (sandwiches) and to particular ingredients (chicken ham or pork). The microbiological information consisted of evidence of a common serotype (*Salmonella* Agona), having a particular genotypic profile (PFGE Profile SAGOXB.0066) which was found in a large

number of cases across Europe, in the production plant of the Irish Food manufacturer and in food outlets across Europe supplied by this manufacturer (at food outlet level, the outbreak clonal group was eventually identified in unopened packs of food produced by the parent company). Taken together, this evidence was used to form a hypothesis that contamination due to this strain (possibly at the level of the parent company) was distributed by means of particular food items through a supply chain to end user food outlets. It was in this way that the infection was transmitted, and the outbreak propagated.

In investigating the root cause of the outbreak, the investigators noted that food was cooked in the plant in a process that involved chicken, bacon, pork and other food types being placed in "continuous cook" ovens on the "low-risk" side. Cooking would take place and the food was then conveyed to the "high-risk" side. The investigators noted that, "*a number of Salmonella isolates identified in the low risk area on product and in the environment between April and July 2008 were forwarded for definitive typing and found to be the unique pulsed field profile SAGOXB.0066/PT39. It appears that there was a high load of S. Agona in the low risk area and to such an extent that it overcame the existing control mechanisms designed to protect the high risk area from material in the low risk area. Such an amount of a single serovar indicated a hygiene failure sufficiently to propagate such an outbreak.*"

When remediation measures were put in place in the affected production plant, the outbreak was controlled.

Without PFGE methods it would have been much more difficult to separate out the outbreak *Salmonella* Agona isolates from non travel-associated endemic isolates across multiple countries. Use of PFGE was instrumental in focussing the investigation towards the likelihood that the outbreak was caused by an internationally distributed commodity, in this case, a food product. PFGE was also used to distinguish between at least one other contemporaneous background Irish *S.* Agona case and the outbreak strain, thus enabling this case to be eliminated from the descriptive and analytical epidemiological investigations. Ensuring that unrelated cases are not included as outbreak cases in analytical studies is particularly important as their exclusion reduces the risk of misclassification (a form of bias), which could alter estimation of the effect size.

The authors of the Outbreak Report say as much when they note that "The detection of the source identified would not have been possible without the use of molecular typing techniques and the sharing of data and co-operation between numerous agencies." [48]

Similarly, a cluster of seven cases diagnosed with *S.* Heidelberg (an uncommon serotype in Ireland) was identified in 2011.[49] In investigating this outbreak, the identification of isolates in reference laboratories in Europe and North America with PFGE profiles indistinguishable from those of the Irish *S.* Heidelberg isolates permitted the recognition of cases which were investigated for possible epidemiological links to the Irish cases. Travel to Tanzania was identified as a common risk factor among cases. Accumulated evidence over a number of years of an association between this serotype and East Africa (among other regions) provided useful circumstantial evidence supporting the hypothesis that the infection was associated with the travel destination. PFGE was important in focussing this investigation towards specific exposures, as the Irish cases had travelled as a group and had shared many exposures throughout their trip making it difficult to establish which was the likely source of infection. In the absence of formal standardisation of molecular typing methods, it would

not have been possible to establish the potential links between these international cases, which might otherwise have been considered to be unlinked.

Unusually in 2010, DT8 was the most common *S*. Typhimurium definitive type detected in Ireland. This was due to the occurrence of an outbreak which was associated with exposure to duck eggs.[50] Prior to 2009, there had only been three cases of this definitive type detected over an eight-year period. The detection initially of a cluster of three *S*. Typhimurium DT8 isolates by the reference laboratory within a one-month period in the latter half of 2009, followed by a further cluster of four cases five months later led to the recognition of a temporally diffuse outbreak of 35 cases which occurred over an 18 month period. In this outbreak, hypothesis generation was based primarily on the classical descriptive epidemiological method of administering a trawling questionnaire; however, particularly strong evidence pointing towards the association between the human cases and duck egg exposure was provided through comparison of molecular profiles of *S*. Typhimurium isolates from implicated duck egg farms with isolates from human cases using both MLVA and PFGE. The work of national veterinary reference laboratory and effective liaison between human and veterinary reference laboratory services was also indispensable in defining the source of this outbreak.

This evidence was key in enabling control measures to be introduced, including the signing into Irish law of new legislation (S.I. No. 565 of 2010), the 'Diseases of Animals Act 1966 (Control of *Salmonella* in Ducks) Order 2010', which now sets down a legal basis for the control of salmonellosis in egg-laying duck flocks in Ireland.

Unfortunately, the identification of clusters by microbiological methods does not guarantee a successful outcome to the subsequent epidemiological investigation. On a number of occasions, outbreak control teams have been established to investigate clusters identified in this manner, but for which no definitive epidemiological link could be established between cases and no source of infection was identified. For example, a temporally-defined but geographically diffuse cluster of *S*. Typhimurium DT193 was investigated in 2009. MLVA was used to define those DT193 isolates occurring that year which were included in the investigation. And in 2009, an outbreak control team was established to investigate a rise in the incidence of *S* Enteritidis PT14b. In neither instance could a definitive epidemiological link be established between the cases and no sources of infection were identified.

Known associations between particular reservoirs and *Salmonella* serotypes has been exploited in source attribution studies.[51] This kind of information is also useful in outbreak investigation as it can give an early pointer of likely vehicles for particular strains for hypothesis generation.

6. Emerging factors

In Ireland, it has become apparent in recent years that overseas travel plays an important role in *Salmonella* epidemiology. It is now estimated that up to half of all notified cases may be travel associated (Table 3). This is broadly similar to the proportions in Finland, Sweden and Norway, all of whom report that more than 70% of their salmonellosis cases are travel-associated and is in contrast to the majority of countries in central and southern Europe who report this to be a largely indigenous disease.[4]

	Number of cases	% of total number of cases	% of cases with known travel history
Indigenous	351	31%	51%
Travel-associated	342	30%	49%
Unknown/not specified	445	39%	-
Total	**1138**	**100%**	**100%**

[Data source: CIDR]

Table 3. Number and percentage Salmonella notifications by Travel history, Ireland 2008-2010

Combining epidemiological information on case travel histories with microbiological information enabled confirmation that *S.* Enteritidis is uncommon among indigenous salmonellosis cases in Ireland, with a high proportion being associated with overseas exposure,[29] while *S.* Typhimurium is clearly the dominant serotype among indigenous cases. This is supported by outbreak surveillance data (Table 4). These combined data have also been exploited in studies such as a recent EFSA source attribution study which suggested that after the risk factor 'travel', pigs may be the most important contributor to human *Salmonella* infections in Ireland.[49]

Serotype	Number travel-associated Salmonella outbreaks	Number indigenous Salmonella outbreaks	Total
S Enteritidis	13	13	26
S Typhimurium	3	37	40
Other Salmonella serotypes	5	24	29
Salmonella serotype not reported	4	10	14
Total	**25**	**84**	**109**

[Data source: CIDR]

Table 4. Number Salmonella outbreaks (family and general) by serotype and travel association, Ireland 2004-2010

7. Conclusion

Salmonellosis continues to be an important global cause of infectious intestinal disease and in developed countries maintains its dominant position as one of the top three commonest causes of bacterial gastroenteritis. Enteric salmonellae are potent outbreak organisms and linking of cases that are part of the same outbreak has been facilitated by the recent increased application of molecular methods of characterisation that allow increasingly

reliable differentiation and discrimination between and within serotypes. The progressive refinement of discriminatory methods permits the ready inclusion (and exclusion) of isolates within outbreaks in such a way that reduces wasteful investigation of unrelated isolates of the same serotype, while identifying more accurately the true extent of outbreaks. This has been assisted by the increasingly rapid turnaround time for identification of such isolates. Long lead-in time of such methods in the past made them more suited to research purposes but the rapidity with which microbiologists can provide results to epidemiologists makes this a real-time method that facilitates investigation and allows more rapid implementation of control measures. However, the most fundamental requirement in the application of laboratory characterisation of isolates to the protection of public health is not the sophistication of the laboratory methods, but open, effective and timely communication between those delivering the laboratory services and those in the public health and food safety domains charged with surveillance and intervention.

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