25 Years on—an update on topics selected from the first volume of the Journal

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**HAEMOPHILUS INFLUENZAE—THE PAST, THE PRESENT AND THE FUTURE**

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In addition to being the 25th anniversary of the Journal of Medical Microbiology, 1993 was also the centenary of the first cultivation of *Haemophilus influenzae* by Pfeiffer. The historical landmarks in *H. influenzae* research have been comprehensively reviewed elsewhere but the most important are described below. The first documented observations of haemophili were by Koch, who described *H. aegyptius* (or *H. influenzae* biogroup aegyptius) from conjunctivitis in 1883. Ten years later, Pfeiffer described and cultured, with the aid of blood-supplemented agar, the bacillus he thought was the cause of influenza and it was officially named *Haemophilus influenzae* by the American Society of Bacteriologists in 1917. In 1931, Margaret Pittman identified capsulate and non-capsulate strains and showed that the former can be subdivided into six capsular types (a–f) distinguishable by serotyping with type-specific antisera. She also made the significant association between *H. influenzae* serotype b (Hib) strains and meningitis around this time, which was very fortuitous as the description of influenza viruses in 1933 could otherwise have left *H. influenzae* as an organism without a disease. The 1950s saw a great deal of work by May and others on non-capsulate *H. influenzae* (NCHi) and its association with respiratory disease. The possibility that these non-capsulate strains had been capsulate at the time of bronchial invasion had been raised and in the first issue of the Journal, in 1968, Turk and Holdaway investigated this possibility. They concluded that capsulate strains played no part in bronchiectasis.

The last 25 years have seen many developments in...
microbiology which have increased our understanding of the pathogenesis and epidemiology of *H. influenzae* disease. The presence of a capsule has been shown to be a major virulence factor of Hib. The host’s protective immune response is targeted against the Hib capsule and this is the basis of an effective vaccine. The genetic basis of capsule production has been characterised extensively and the mechanism whereby strains lose the ability to produce capsule is now established. Given these advances, if we were to ask a similar question today to that which Turk and Holdaway asked in their 1968 paper—i.e., “Are non-serotypable *H. influenzae* from bronchictasis derived from capsulate strains?”—how would our approach differ from theirs?

The laboratory identification of *H. influenzae* has changed very little in 25 years. Growth around commercially available disks containing factors X and V is the principal test for identification, and iridescence on Columbia agar supplemented with haemin, NAD and antisera has provided a simpler method of demonstrating the presence of capsule than the traditional Levinthal’s agar. The capsular serotyping method described in 1931 is still used by the majority of laboratories which serotype *H. influenzae* isolates. However, this is one area in which modern technology is making a contribution. The polymerase chain reaction (PCR) has changed how the capsular type of *H. influenzae* is both determined and defined. The gene clusters responsible for capsulation (cap) in each of the six capsular types have been cloned. Capsule type-specific regions of each of the cap loci have been sequenced and primers designed which can be used for amplification in a PCR.

One of the major advances of the past 10 years has been the unravelling of the structure of cap. The salient features are shown in fig. 1. The basic structure consists of three regions (1, 2 and 3). Region 2 is specific to each capsular type and is flanked by regions 1 and 3 which share sequence homology with the equivalent regions in all six capsular types. Region 1 contains the *bexA* gene which is essential for export of capsular material and hence for the expression of capsule on the cell surface. In type b strains the whole unit is flanked by copies of an insertion sequence element (IS1016) and is usually present as a duplication with a 1·2-kb deletion leaving only one functional *bexA* gene. Homologous recombination occurs at a frequency of 0·1–0·3% and reduces this duplication to the basic unit; however, this also results in the loss of the single functional *bexA*. The resultant strains are, therefore, genetically type b but are capsule-deficient (Hib strains) and are non-serotypable by traditional serotyping methods.

The use of PCR primers for the capsule type-specific region enables the detection of type b-specific DNA in both Hib and Hib- strains. Primers designed within *bexA* detect the intact *bexA*, which is necessary for capsule production. Therefore, Hib and Hib- strains can be distinguished by the amplification of the former with these *bexA*-derived primers. Hib- strains can be confirmed with PCR primers derived from DNA flanking *bexA*: *bexB* (in region 1) and IS1016 sequences. These primers amplify one product in Hib- strains (corresponding to the deleted *bexA* gene) but two products of different sizes in Hib strains (from the intact *bexA* and the deleted *bexA*) (fig. 2). Therefore, PCR can be used to determine whether a non-serotypable strain is derived from a type b capsule strain (i.e., is a Hib strain).

The last 20 years have also seen advances in bacterial typing techniques. Some of those which have been advocated for *H. influenzae* include biotyping, outer-membrane protein (OMP) subtyping, lipopolysaccharide analysis, whole-cell polypeptide profiles, restriction enzyme analysis, ribotyping and randomly amplified polymorphic DNA analysis. None of the current methods is very useful for Hib, as 80% of all strains are indistinguishable; it has a very clonal population genetics structure. NCHi are much more heterogeneous and many techniques have been applied successfully to epidemiological investigations of putative outbreaks of infection. Most of these are electrophoretic techniques that give banding patterns which can be compared by eye or by computer. The large number of different banding patterns obtained means that these techniques are generally very good at discriminating between strains but it must be remembered that these are purely comparative techniques and the use of epidemiologically unrelated control strains is vital for interpretation of the results.

The differences between capsule and non-capsulate strains, in terms of the diseases with which they are associated, were well established in the 1930s–1950s. Hib is the major pathogen but it is important to remember that although strains of other serotypes and NCHi may be less virulent, they can cause invasive disease. A recent study of non-capsulate strains from invasive disease in neonates and children asked whether these strains played a part in invasive disease and if such strains were actually capsule-deficient type-b strains. Two of 21 strains, examined by the molecular techniques described above, were shown to be Hib. Therefore, we have almost come full circle in 25 years, asking about the role of Hib- strains. In addition to the morbidity associated with NCHi as a cause of otitis media and exacerbations of chronic bronchitis, NCHi have also been associated with several hospital-acquired outbreaks of respiratory infection in the UK, the financial cost of which is not insignificant.

Although serious infections and hospital-acquired carriage or infection can be attributed to NCHi, Hib is still the pathogen which generally concerns us most. The single most important contribution to the management of these infections over the last 25 years has undoubtedly been the introduction of an effective vaccine against Hib and the last few years have seen dramatic reductions in the incidence of Hib meningitis in countries where the vaccine has been introduced.
Fig. 1. Structure of \textit{cap} in Hib and Hib\textsuperscript{–} showing targets for amplification by PCR. I (top row), basic structure; II, basic unit flanked by IS\textsubscript{1016}; III, duplication of basic unit with single functional \textit{bexA} in Hib; IV, single copy of basic unit with detected \textit{bexA} in Hib\textsuperscript{–}.

Fig. 2. Differentiation of Hib and Hib\textsuperscript{–} by the PCR; b, Hib; b\textsuperscript{–}, Hib\textsuperscript{–}; m = 1-kb size marker. The strains were amplified with primers for the capsule-specific type b region (b primers); primers within \textit{bexA} (v primers) or primers for IS\textsubscript{1016} and \textit{bexB} (is primers); Hib\textsuperscript{–} gives no product with v primers and lacks the larger product with is primers.

The impact of the Hib conjugate vaccine, introduced into the routine immunisation programme in the UK and Ireland in October 1992, has been dramatic.\textsuperscript{13} The number of cases of Hib meningitis and septicaemia in children < 1 year old, notified to the PHLS Communicable Disease Surveillance Centre, decreased in 1993 to 16\% of the number for previous years (38 compared with a mean of 233 for each of the preceding 3 years). However, some host populations respond differently to the different vaccines, e.g., Alaskan eskimos responded poorly to a Hib vaccine that was highly effective in Finland, so it is important to monitor the effect of the vaccine. A study to monitor Hib vaccine failures, set up by the British Paediatric Surveillance Unit, has been in progress since October 1992. Since the introduction of the vaccine in the British Isles there have been 17 reports of "true" vaccine failures, i.e., where Hib disease has occurred when protective immunity should have developed (at least 1 week after a child aged < 1 year has received at least two doses of Hib vaccine, or at least 3 weeks after a single dose of Hib vaccine in children aged ≥ 1 year). There have also been 47 "apparent" vaccine failures, when disease occurred after a single dose of vaccine and before protective immunity could have developed. With the PCR techniques described above, none of the strains from these patients was Hib\textsuperscript{–}. However, the vaccine is not expected to have any effect on infection with capsular types other than type b or non-capsulate strains, which are important causes of serious disease in other parts of the world and \textit{H. influenzae} (Hib and NCHi) is expected to remain an important cause of invasive disease in adults. Before the introduction of Hib immunisation in the UK, c. 10\% of invasive disease was caused by NCHi and 1.7\% by capsulate strains of serotypes other than b.\textsuperscript{13} This is even more striking in developing countries where other serotypes (notably a) and NCHi are a significant cause of meningitis and pneumonia.\textsuperscript{14, 15} Therefore, a vaccine against serotypes other than b and NCHi would be desirable.

So, what does the future hold? Will there be a vaccine against non-capsulate strains? NCHi are very heterogeneous so it will not be as straightforward as the Hib vaccine story. Several immunogenic targets have been investigated and there has been some work on OMP-directed vaccines. Many potential targets,
such as OMP P2, are too specific (strain specific) but several other proteins which are conserved between strains have also been advocated. Other possibilities include iron-regulated OMPs such as transferrin-binding proteins (TBPs), but studies of antigenic relationships amongst TBPs have so far been performed with only a very limited number of strains (K. J. Towner, personal communication).

In the last 25 years, much has been learnt about the genetic structure of capsule production and the pathogenicity of NCHi and there is now an effective vaccine against Hib. The vaccine has reduced the concerns of the 1970s and 1980s about the increasing antibiotic resistance in this organism. However, it remains important to remember that H. influenzae other than type b can cause problems and the organism should not be forgotten simply because Hib vaccine has been introduced.

**Bordetella pertussis—25 years on**

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The paper by Holt and Spasojevic in the first issue of the *Journal of Medical Microbiology* provides a useful reference point for an update on *Bordetella pertussis* and pertussis research. It incorporated three topics—the search for *B. pertussis* protective antigens, antigenic variation and animal models of pertussis—which form the basis of this brief and, therefore, selective review. The theme of their paper was to examine the role of particular surface antigens in the protective potency of *B. pertussis* cell suspensions in the mouse intracerebral challenge test. The whole-cell vaccines in use at that time were known to be reactogenic and there was much interest in identifying the protective antigens in order to separate them from the toxic components of the bacterium. In the last 25 years, various purified *B. pertussis* antigens have been shown to be protective in animal models and are candidate vaccine components, but the search goes on. Holt and Spasojevic were able to induce changes in the surface antigens of *B. pertussis* by growth on different media. Much is now known about the molecular mechanisms of this and related variation processes in *B. pertussis* but their significance is obscure. The mouse intracerebral challenge test may seem rather inappropriate for estimating the protective efficacy of vaccines for a respiratory disease of man, but the test is still in use today. All animal models of pertussis have their shortcomings but the recently described coughing rat model may provide new opportunities for studying pathogenic mechanisms and vaccine efficacy.

Twenty-five years ago, the epidemiology of pertussis in the UK was similar to that seen today, with a low level of annual notifications and the disease controlled by a high uptake of whole-cell vaccine. In the intervening period, there was a dramatic change to this picture. Fig. 3 shows the steady decline in the incidence and severity of pertussis in England and Wales, with the introduction of immunisation in the 1950s. However, from 1974, following widely publicised concerns over the safety of pertussis vaccines, uptake rates fell sharply, from > 80% to 30% in some areas. The value of pertussis vaccination, especially in view of the low incidence of the disease at the time, was fiercely debated. As can be seen, this was followed by major epidemics of pertussis around 1978 and 1982, with younger, unvaccinated children being most affected. As vaccine uptake has recovered, control has once again been established. The cyclical nature of the disease, with epidemic peaks every 3–4 years, is also evident from fig. 3 but it is not clear why the frequency of these cycles has not been altered by the introduction of immunisation or by recent changes in vaccine uptake. One suggestion is that whole-cell vaccines provide better protection against the disease than against infection and that vaccination has had little effect on the prevalence of *B. pertussis* in the population.

Whole-cell vaccines are undoubtedly reactogenic and different preparations may vary. Swelling and redness at the site of injection are common. More severe reactions such as high fever, persistent crying and seizures are less frequent, but all are transient. An alleged association between pertussis vaccination and permanent neurological damage or death has never been demonstrated conclusively and, if these events do occur, they must be very rare. In spite of the reactogenicity of the vaccine, it is generally agreed that the benefits of controlling pertussis and its associated hazards far outweigh the risks.

It is interesting to note that the proportion of adults with pertussis is increasing steadily. In Scotland, for example, 10% of all notified cases are now in adults (> 15 years old) compared with 2% 25 years ago. Several factors may be contributing to the increase, such as improved diagnosis and immunity in adults, but the non-vaccinated cohort from the 1970s is now entering this age group.

The problems with pertussis vaccine have been mirrored in other parts of the world but, where there is a high vaccination rate, the disease is controlled. However, pertussis still causes severe morbidity and mortality in unvaccinated communities with 60 million cases and > 0.5 million deaths per annum estimated in 1986. Fortunately, increasing global immunisation coverage by schemes such as the WHO Expanded Programme on Immunization is steadily reducing these figures.

Whatever the rights and wrongs of the pertussis vaccine debate, it provided a major stimulus for pertussis research and for the development of new pertussis vaccines devoid of side effects. The aim was to prepare defined acellular vaccines which would be fully characterised in terms of their antigenic composition and in which the toxic components of whole
cells would be absent or inactivated. A less reactogenic vaccine would also promote the acceptability of pertussis vaccination worldwide and could be used to boost the waning immunity of adults. Another aim was to improve protective efficacy and to prevent infection as well as disease, an essential requirement for any future pertussis eradication programme. In the search for pertussis protective antigens, certain problems have had to be addressed. The pathogenesis of pertussis is complex and identification and characterisation of the virulence factors of *B. pertussis* have been troublesome. A major factor has been the shortcomings of the various animal models available, although intracerebral and intranasal challenge tests in mice have provided much useful information.²⁸

Twenty-five years ago, several biologically-active factors of *B. pertussis* had already been described. The idea was just beginning to emerge that some of these, such as the histamine-sensitising factor, leucocytosis-promoting factor, heat-labile adjuvant and a protective antigen for mice, were in fact one and the same, a component now known as pertussis toxin (PT).²⁹ In recent years, remarkable insight into the virulence properties of *B. pertussis* has been achieved by the application of modern molecular techniques. In fact, due largely to the pioneering work of Weiss and Falkow,²⁹ *B. pertussis* has become almost a model system for this approach to the study of bacterial virulence. Their method for transposon mutagenesis with a suicide plasmid to deliver transposon Tn5 into the *B. pertussis* chromosome has led to the identification of several genes encoding virulence factors or regulating the expression of virulence. Many of these have now been cloned, sequenced and expressed in other bacteria. In addition, strains engineered with precise mutations have been used to investigate the effect of loss of individual factors on the ability to colonise and cause disease in experimental animals.

Current information on *B. pertussis* virulence factors is summarised in table I. Several toxins and adhesions for different mammalian cells and tissues have been identified and purified.³¹ It is still not clear why (or whether) *B. pertussis* needs all of these and what role they play in pathogenesis, but the results suggest that the organism has a number of alternative strategies for ensuring its survival and propagation within the host population.³² In some cases, these factors have been protective both in mouse models of infection and in recent field trials of acellular vaccines in human infants.

The factor that has received most attention as a vaccine constituent is PT. It has a remarkable range of activities *in vitro* and *in vivo*; e.g., it is an ADP ribosyltransferase, like some other bacterial toxins, but it is also a haemagglutinin and a T-cell mitogen. Furthermore, PT causes leucocytosis, which is a characteristic feature of pertussis, and enhancement of insulin secretion and inhibition of adrenaline hyperglycaemia which have also been noted in the disease in man. In toxoided form, PT can be protective but, untoxoided, it has a range of potentially harmful effects on experimental animals, such as histamine sensitisation and potentiation of anaphylaxis, and it is lethal for mice in µg amounts. PT is a subunit toxin with an enzymic moiety, the S₁ or A subunit, and a complex B subunit responsible for binding to target cells and inserting the S₁ subunit into the cytoplasm.³³ The haemagglutinating and mitogenic activities of PT are due to the B subunit acting on the target cell surface but most of the other effects result from activity of the S₁ subunit within the target cell. There it interferes with particular G proteins and trans-
Table I. Virulence factors and protective antigens of *B. pertussis*

<table>
<thead>
<tr>
<th>Virulence factor and synonyms</th>
<th>Possible role in natural infection</th>
<th>Protective activity in mouse*</th>
<th>man</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pertussis toxin</strong></td>
<td>Leucocytosis-promoting factor, histamine-sensitising factor, islets-activating protein etc.</td>
<td>Adhesion, inhibition of immune effector cells, leucocytosis promotion, other systemic toxic effects</td>
<td>+</td>
</tr>
<tr>
<td><strong>Filamentous haemagglutinin</strong></td>
<td>Agglutinogens 2 and 3</td>
<td>Adhesion</td>
<td>+</td>
</tr>
<tr>
<td><strong>Fimbrial or serotype antigens</strong></td>
<td>69-kDa Outer-membrane protein Pertactin</td>
<td>Adhesion</td>
<td>+</td>
</tr>
<tr>
<td><strong>Adenylate cyclase toxin</strong></td>
<td>Cyclolysin</td>
<td>Inhibition of immune effector cells</td>
<td>+</td>
</tr>
<tr>
<td><strong>Heat-labile toxin</strong></td>
<td>Dermonecrotic toxin</td>
<td>Local damage</td>
<td>-</td>
</tr>
<tr>
<td><strong>Tracheal cytotoxin</strong></td>
<td>Lipopolysaccharide</td>
<td>Ciliary damage</td>
<td>?</td>
</tr>
<tr>
<td><strong>Endotoxin, lipo-oligosaccharide</strong></td>
<td></td>
<td>Fever</td>
<td>-</td>
</tr>
</tbody>
</table>

*Protection against challenge with *B. pertussis* by the intranasal route. Only PT is protective by the intracerebral route.*

Table II. Examples of defined acellular pertussis vaccines

<table>
<thead>
<tr>
<th>Vaccine type</th>
<th>Composition</th>
<th>Toxoiding process</th>
<th>Stage of development</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st generation</td>
<td>Ptd and FHA as main components, co-purified from culture supernate</td>
<td>Formaldehyde</td>
<td>Routine use in Japan since 1981 Licensed as booster in USA, 1991 Swedish phase 3 trials 1986</td>
</tr>
<tr>
<td>2nd generation</td>
<td>Individually-purified and chemically-inactivated components</td>
<td>Formaldehyde</td>
<td>Swedish phase 3 trials 1986</td>
</tr>
<tr>
<td>3rd generation</td>
<td>Containing recombinant proteins from <em>B. pertussis</em> or other organisms</td>
<td>Genetic</td>
<td>Phase 3 trials in progress</td>
</tr>
</tbody>
</table>

Based on a table from Hewlett and Cherry.27

Membrane signalling, resulting in a range of different effects on different host cells. PT has now been rigorously purified and, in chemically-toxoided form, is one of the major components in almost all of the new acellular vaccines.

At around the same time as these purification and characterisation studies other investigators identified the genes for PT expression and sequenced the PT operon.24,25 This breakthrough enabled Rappuoli and co-workers to map the important regions of the molecule such as those in the S1 subunit responsible for enzymic (toxic) activity and immunogenicity in terms of the B-cell and T-cell epitopes required for protection. With this information, they were then able to use site-directed mutagenesis to alter the molecule. It was found that by changing just two amino acids (Arg 9 → Lys and Glu 129 → Gly), they were able to eliminate toxic activity completely while retaining immunogenicity. This genetic toxoid is more immunogenic than conventional chemical toxoids and highly protective in mice because it is structurally and antigenically indistinguishable from the native toxin. Chemical detoxification with agents such as formaldehyde damages the structure and epitopes of the molecule and yet may still allow reversion to toxicity. By contrast, the genetic toxoid is irreversibly toxoided.

The first of the defined acellular vaccines29—37 were developed by Sato et al.36 in the late 1970s. Such "first generation" pertussis vaccines (table II) contain toxoided PT (Ptd) and filamentous haemagglutinin (FHA), one of the adhesins, as their main components. However, because of the way in which these vaccines are prepared, they also contain smaller amounts of other components as contaminants. Toxoiding of PT
is usually done with formaldehyde. Several such vaccines with different compositions have been used in place of whole-cell vaccines in Japan since 1981. All available evidence indicates that they are effective and less reactogenic than the whole-cell products but they have never been subjected to rigorous clinical trials in Japan. "Second generation" vaccines contain highly purified ingredients and range from a monocOMPonent PTd vaccine to a multicomponent vaccine containing PTd with the adhesins FHA, agglutinogens (AGGs) and pertactin.

Two of these vaccines, a monocOMPonent PTd and a two-component PTd + FHA vaccine were used in a large, double-blind, placebo-controlled field trial in 1986–1987 in Sweden,39 a country where pertussis was prevalent as a result of a policy not to immunise against the disease. Their reactogenicity was low, as expected, but efCacy results were disappointing. They were less protective against culture- or serologically-confirmed pertussis than would have been expected for a whole-cell vaccine. However, both vaccines gave good protection against severe disease and provided evidence that PTd alone or with other antigens could make effective vaccines.

Further trials are now in progress in Sweden and elsewhere of other vaccine formulations, examples of which are shown in table II. One particularly promising vaccine is a "third generation" product containing the recombinant PTd (genetic toxoid) mentioned above, and phase 3 trials are in progress in Italy. If the results, expected in 1995–1996, are good, there may be little impetus for the development of further generations of pertussis vaccines, such as those containing immunogenic peptides or live attenuated organisms.

In addition to the search for protective antigens, the pertussis paper in the first issue of the Journal was concerned with antigenic variation. B. pertussis is, potentially, a highly adaptable organism.32 It can undergo serotype variation in which expression of surface antigens is altered by mutational events. It can also alternate between virulent and avirulent forms either by mutation (phase variation) or by a freely-reversible phenotypic change (antigenic modulation) in response to in-vitro growth conditions. The mechanisms of these variation processes are starting to become clear.30 For example, the organism has mutational "hot spots" in certain regions of the genome that promote frameshifs to allow serotype variation and phase variation at fairly high frequency. Antigenic modulation is controlled by a two-component sensor-regulator system with homology to systems in other bacteria. A surface-located sensor protein, BvgS, is thought to detect environmental changes, e.g., in temperature or ionic conditions, and transmit a signal to the response regulator BvgA, a cytoplasmic DNA-binding protein. Under normal growth conditions, BvgA activates transcription of the well-characterised virulence genes (vir-activated genes), either directly or indirectly, and at the same time represses another set of genes (vir-repressed genes) whose products have not yet been characterised.

It is not entirely clear why B. pertussis needs these variation processes. Serotype variation is presumably a strategy for evading the host immune response and vaccine-induced immunity appears to have a serotype-specific component,3 as originally proposed by Preston.41 With regard to antigenic modulation, many pathogens can detect changes in their environment and use the signals to regulate virulence factor expression at different stages in the disease process or at different sites in the host. But what are these stages or sites for B. pertussis? Until recently, it was thought that B. pertussis resides exclusively on the ciliated epithelium of the human respiratory tract but recent evidence suggests that it may have an intracellular stage.43 It is capable of invading and surviving within mammalian cells, e.g., macrophage and tissue culture cell lines, perhaps again to avoid the host immune response. Switching off the expression of virulence genes is also a way of adapting to life outside the host, but no alternative host or site has been reported for B. pertussis. All Bordetella spp. are generally regarded as obligate parasites of the respiratory tract of mammals and birds but recent evidence has changed this view. B. parapertussis was thought to be purely a parasite of man, like B. pertussis, but has now been isolated from healthy and pneumonic sheep.43 In addition, B. bronchiseptica can survive and grow in natural waters.44

The final part of this update concern'$a new animal model of pertussis, namely the coughing rat model,18,20 which is being developed in our laboratory. Study of the cough is important because of its central role in the disease process. Many of the respiratory complications and central nervous system disturbances associated with pertussis are thought to result from the severe coughing.25 The model is based on earlier observations on the induction of coughing in rats infected intrabronchially with B. pertussis.53 In our experiments, the rats are infected with B. pertussis encaiced in fine agarose beads and the intermittent paroxysmal coughing is recorded and analysed with sound-activated tape recorders. The animals also show leucocytosis and, in young rats, significant retardation of weight gain. Wild-type or phase I B. pertussis strains all induced coughing paroxysms whereas a phase variant, lacking all of the major virulence factors, did not. More significantly, a transposon-insertion mutant lacking only PT did not induce coughing. Therefore, it appears that of all the virulence factors produced by B. pertussis, PT appears to have a central role in the disease process, as suggested by Pittman57 some years ago. Since the rat is the only conveniently accessible laboratory animal in which B. pertussis produces paroxysmal coughing, the model has much potential for further study of the mechanisms of cough production and immunity in pertussis, including the investigation of virulence factors and acellular vaccines.
I thank Elizabeth Miller for providing the figure and A. Wardlaw for helpful comments on the manuscript.

**Escherichia coli infection in farm animals—25 years of progress**

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During the 1960s, the role of *Escherichia coli* as an enteric pathogen in farm animals was clearly established. The work of Smith and his co-workers redefined Koch’s postulates to take account of developments in microbial genetics. The mechanisms of adherence of *E. coli* and the biochemical effects of its toxins are now being clarified with the advent of sophisticated molecular and cellular biological tools. The purpose of this brief review is to highlight the developments in the veterinary field since the first publication of this *Journal* in 1968.

**The *E. coli* toxins**

Heat-labile enterotoxin (LT) exists as a high-mol. wt protein that resembles cholera toxin (CT) both pharmacologically and immunologically and is not inactivated by heating at 60°C. A wide variety of different *E. coli* serogroups of porcine origin produce LT; these serogroups usually possess the F4(K88) fimbrial adhesin. The LT of porcine origin has common and unique antigenic determinants when compared with LT produced by human isolates of *E. coli*. Another enterotoxin (LT2) was detected in *E. coli* isolates from cattle and, while it shared many biological properties with LT1, it was not neutralised by antiserum to CT or LT1. LT2, like LT1, activates adenylate cyclase in eukaryotic cells but, in contrast to LT1 whose genes are plasmid encoded, it genes appear to be on the chromosome.

Heat-stable enterotoxins (ST) are low-mol. wt proteins that resist heating at 100°C for 30 min. Two types of ST have been described. STa, which is methanol soluble, is active in the gastrointestinal tract of calves, sheep and neonatal mice and is usually produced by *E. coli* strains that express the fimbrial adhesins F5(K99), F41 and F6(987P). STas from *E. coli* isolates from man, cattle and pigs have been purified and, although they share similar chemical and biological activities, there is slight molecular heterogeneity. STb, which is methanol insoluble, induces intestinal secretion in both the newborn and the weaner pig and has no apparent toxicity in mice. It is usually associated with LT + *E. coli* strains that express the F4 fimbrial adhesin, although other combinations of toxin and fimbriation have been described.

Verocytotoxigenic *E. coli* (VTEC) strains were first described in 1977. The cytotoxin, similar to Shiga toxin, destroys Vero cell monolayers and possesses two polymorphs VT1 and VT2 (also called SLT1 and SLT2). VTEC have been isolated from diseased calves and pigs and in the latter species they are often associated with oedema disease. Although VT from porcine *E. coli* isolates share considerable genetic homology, as demonstrated by hybridisation with a VT2 probe, the cytotoxin activity is not neutralised to the same extent by antitoxin to VT2. Differentially described as VT2e, this toxin differs from VT2 in being inactive on HeLa cells, more heat-labile and not being phage-mediated like VT1 and VT2. DNA transfer experiments showed that the verocytotoxicity of oedema disease strains could be transferred to *E. coli* K12 and that pigs inoculated with the transconjugant developed the clinical and pathological lesions of the disease.

Cytotoxic necrotising Factor (CNF) was first described in *E. coli* isolates from man and has been detected subsequently in several *E. coli* serotypes isolated from farm animals. CNF is a heat-labile cytotoxin that causes multinucleation and giant cell formation in Vero, HeLa and CHO cells and necrosis when injected intradermally into rabbits. Although CNF* E. coli* strains have been detected in a wide range of serogroups from different clinical conditions in farm animals, most isolates belong to a small range of serogroups. A second type of CNF, which is only partially neutralised by antitoxin to CNF1 has been described, and while both types share considerable genetic homology, CNF1 is encoded chromosomally and CNF2 is encoded on the transmissible Vir plasmid.

**Fimbrial antigens and putative colonisation factors**

Several specific fimbrial adhesins, whose attachment to erythrocytes and epithelial cells, or both, is not impaired by D-mannose, have been shown to be important virulence factors in the pathogenesis of diarrhoea in farm animals. Some, such as F4, F5, F6 and F41 fimbriae, were identified many years ago while others (table III) have been recognised more recently. Whether some of the more recently identified surface antigens are true colonisation factors awaits clarification and there is also an urgent need for a standard nomenclature and a reference centre. Colonisation factors are associated with ETEC, VTEC and bacteraemic *E. coli* strains and the genes may be encoded on plasmids or the chromosome. Whereas some fimbrial antigens are expressed in vivo, the antigen may not be expressed, or only partially expressed, in culture.

Some pigs show genetic resistance to intestinal colonisation by ETEC, thus F4* and F107* strains do not adhere to isolated brush border preparations from all piglets. F41 fimbriae, although antigenically distinct from the F4 antigen, share considerable gene sequence homology in those genes associated with processing the antigen. Likewise, some fimbriae often occur in association with others, e.g., F5 with F41, and Cs31a with F165 and F5 antigens.

A novel class of *E. coli* surface structures, “Curli”,
Table III. Recently described putative adhesive fimbrial antigens

<table>
<thead>
<tr>
<th>Antigen designation</th>
<th>O group</th>
<th>E. coli type/ disease syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>F42^61</td>
<td>8</td>
<td>ETEC</td>
</tr>
<tr>
<td>F17^21</td>
<td>8, 9, 15, 78, 101, 86</td>
<td>ETEC/septicaemia in cattle</td>
</tr>
<tr>
<td>F107^82</td>
<td>139, 138, 141</td>
<td>PWD (porcine VTEC)</td>
</tr>
<tr>
<td>F165^83</td>
<td>115, 78, 9, 101, 15</td>
<td>Septicaemia in pigs</td>
</tr>
<tr>
<td>CS31a^64</td>
<td>7, 117, 4, 18, 149</td>
<td>Septicaemia in cattle</td>
</tr>
<tr>
<td></td>
<td>18, 17, 78, 117, 23</td>
<td>Septicaemia in cattle</td>
</tr>
<tr>
<td></td>
<td>134, 9, 157, 87, 161</td>
<td>Septicaemia in cattle</td>
</tr>
<tr>
<td>M326^61</td>
<td>65</td>
<td>Porcine VTEC</td>
</tr>
<tr>
<td>8813^86</td>
<td>25, 108, 138, 141, 147, 157</td>
<td>Porcine ETEC</td>
</tr>
<tr>
<td>C12I35^71</td>
<td>20, 153, 78, 9</td>
<td>Diarrhoea in cattle</td>
</tr>
<tr>
<td>F11^89</td>
<td>1, 2, 78</td>
<td>Septicaemia in poultry</td>
</tr>
</tbody>
</table>

which are morphologically and biochemically distinct from other surface appendages, have been described recently on strains isolated from bovine mastitis.\(^7^3\) Curli are fibronectin- and laminin-binding fibres, expressed at temperatures below 37°C when grown on CFA medium. Whether Curli are true virulence factors is unclear, but they share homology with similar thin aggregative fimbriae (SEF17) found on salmonellas.

E. coli disease in farm animals

Colibacillary diarrhoea and toxaemia are well described but the pathogenesis of porcine post-weaning diarrhoea (PWD) is more complex.\(^7^2\) PWD may be attributed to weaning-associated factors interacting with the organism: these include stress at weaning, decreased gastric pH and the lack of lactogenic immunity. Rotavirus, often in conjunction with haemolytic E. coli, has also been associated with PWD, as has hypersensitivity of the intestinal mucosa to dietary antigens. However, the one unifying finding is the marked increase in numbers of haemolytic E. coli that occurs 2–7 days after weaning.

Diseases caused by EPEC

E. coli strains that attach intimately to enterocytes of the intestinal mucosa with loss (effacement) of microvilli are known at attaching and effacing E. coli (AEEC). The formation of the characteristic cup and pedestal, which involves the eae gene, has been reviewed.\(^7^3\) Many strains of AEEC, which also produce VT, have been isolated from a wide range of clinical conditions and the characteristic lesions and blood-stained diarrhoea have been reproduced experimentally.\(^7^5\) A survey of E. coli cultures\(^7^4\) found that 4.7% of 3595 porcine isolates, 2.8% of 1383 bovine isolates and 61% of 407 ovine isolates produced VT. A longitudinal study of E. coli in two cohorts of calves found that 91 of 171 isolates were VTEC and 20–80% of the animals excreted VTEC at any one time, often in association with diarrhoea.\(^7^5\) Examination of 296 E. coli isolates from calves with diarrhoea\(^7^6\) found that 70 hybridised with the eae probe and, of these, 50 hybridised with the VT1 probe, one with the VT2 probe and three produced both VT1 and VT2. Ten eae positive isolates gave negative results with both VT probes.

Zoonotic aspects of VTEC production

Verocytotoxin-producing E. coli, frequently belonging to serogroup O157, have been found to be associated with a number of disease syndromes in man, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Such strains have also been isolated from the faeces of cattle by several investigators,\(^7^7\) and the frequent association between VTEC infection in man and the consumption of beef and dairy products suggests that cattle are a reservoir of VTEC. Following an epidemiological investigation of two sporadic cases of HUS associated with milk, VT-producing isolates, which included E. coli O157, were identified in 13 (8.4%) of 154 healthy dairy cattle. E. coli O157 has also been isolated from cattle in England\(^7^8\) and in Scotland the organism was isolated from scouring calves.\(^7^9\)

In cattle infected with E. coli O157, the organisms are usually present in the faeces in small numbers. The extent of the bovine reservoir is unknown. However, with more sensitive techniques of isolation, Chapman found that infection was widespread in one herd associated with milk-borne infection (personal communication).

Systemic colibacillosis

Systemic colibacillosis occurs frequently in calves, lambs and poultry although it is less frequent in pigs. Bacteraemic strains of E. coli pass through the mucosa of the alimentary or the respiratory tract and enter the blood stream which may result in either a generalised or localised infection. Bacteraemic E. coli strains are able to resist the host’s defence mechanisms and are able to survive in blood and peritoneal fluid.\(^7^2\) The genetic determinants for serum resistance and colicin V production are closely linked. Also, plasmid genes associated with Col V encode a specific high affinity
iron-uptake mechanism which consists of aerobactin, a hydroxamate siderophore and an inducible outer-membrane protein that acts as a receptor for the ferric-aerobactin complex. A good correlation has been shown between aerobactin production and invasiveness of *E. coli* isolates from animals.

Another independent plasmid has been shown to be associated with a lethal toxin, CNF2, and the production of a surface antigen, the genes of which hybridise with F17 probes. The persistence of these plasmid-bearing *E. coli* in the avian respiratory tract may be associated with the presence of pili such as the putative CS31a and F165 adhesin which enable them to adhere to epithelial cells and, recently, F11 fimbriae have been identified on many isolates.

**E. coli vaccines**

*E. coli* disease in farm animals is an important cause of economic loss and several vaccines are available commercially. Vaccines based on purified fimbrial antigens and toxoids have been shown experimentally to provide good passive protection to neonatal animals against *ETEC* infections.

For pigs, toxoid VT2e vaccines have been developed for control of oedema disease and PWD. New approaches to disease prevention are being developed, particularly the use of toxoid and fimbrial antigen mixtures administered with novel adjuvants or immunostimulating complexes. Additionally, recombinant DNA technology is being used to develop rationally attenuated live strains and multivalent vectors.

However, it is perhaps salutary to point out that, despite all the advances made in our understanding of *E. coli* as a pathogen, a recent survey of *E. coli* isolates from farm animals in the UK demonstrated that the common serotypes and virulence determinants were the same as those recorded 25 years ago.

**Staphylococcal aggressins and disease—the last 25 years**

C. G. Gemmell

*Department of Bacteriology, University of Glasgow Medical School, Royal Infirmary, Glasgow*

*Staphylococcus aureus* strains may produce various extracellular substances including the enterotoxins (of which there are six serotypes), toxic shock syndrome toxin and exfoliative toxins (of which there are two serotypes) besides the classical haemolysins (of which there are four types), leukocidins and other exoenzymes which may themselves be intrinsically toxic. Much of this information has been garnered in the last 25 years during which time three toxin-mediated disease syndromes have been recognised. This short review provides more details of how *S. aureus* is still showing new aspects to its pathogenicity for man more than 100 years after its discovery by Alexander Ogston.

The first of these new disease syndromes was described by a dermatologist, Lyell, in Glasgow in 1969 as scalded skin syndrome, which usually affects neonates causing a characteristic blistering of the skin. Phage group II strains of *S. aureus* are most often associated with disease although other phage types can be implicated. It is of interest that the same strain may cause *impetigo contagiosa* in older siblings indicative of important differences in host response related to age. Clinical characteristics of this syndrome are summarised in table IV.

Soon after its description, several attempts were made to attribute the skin blistering to a biological product of the staphylococcus, and although α-haemolysin was first implicated, subsequently a new toxin called epidermolytic toxin or exfoliatin was described. This toxin causes sub-epidermal cleavage and can reproduce one of the main characteristics of the disease in neonatal mice. Exposure of skin to exfoliatin results in significant changes at the ultrastructural as well as histological level. Electron-microscopy has shown that the individual cells of the *stratum granulosum* are attached to each other by specialised cell membrane thickenings called desmosomes and the spaces between the cells are filled with material containing small translucent bubbles. The toxin causes these bubbles to disappear followed by a widening of the intercellular gap and splitting of the desmosomes forming a cleft between the cell layers. At a molecular level, the toxin is probably a serine protease. It is now clear that there are two serological varieties of exfoliative toxin, one of which, (ETA) is a heat-stable protein of 30 kDa whose synthesis is chromosomally controlled; the other (ETB) has almost the same molecular size but is heat labile and plasmid controlled. Antibodies to both toxins can be detected during convalescence.

Chronologically, the next major development in staphylococcal disease came almost 10 years later with the discovery of large numbers of cases of septic shock with a morbilliform rash, desquamation and multiple organ failure related to use of a particular tampon by young menstruating females in certain parts of the USA. The Centers for Disease Control has produced diagnostic criteria for toxic shock syndrome (TSS) and these are described in table V. It was significant that these cases occurred initially in Minnesota and Wisconsin since the discovery of a new staphylococcal toxin, named either enterotoxin F or pyrogenic toxin, could be attributed to the presence of two prominent research groups in those two states. The new toxin was shown to cause vomiting in monkeys and structural and serological evidence suggested that it was different from enterotoxins described previously. In contrast, attributes of streptococcal pyrogenic toxin appeared to resemble those of the new toxin and it was felt that the new toxin could be a relative. After some debate, a consensus view has prevailed recognising the toxin as toxic shock syndrome toxin-1 (TSST-1).

So far only one species of TSST-1 has been described and its biological properties include the capacity to
induce fever directly or indirectly via cytokines from macrophages, to act as a specific mitogen for T cells, to enhance host susceptibility to endotoxin, to suppress immunoglobulin synthesis and to impair tumour necrosis factor-induced chemotaxis of polymorphonuclear leucocytes.  

Although the original outbreak was closely associated with menstruation, non-menstrual TSS has been described almost as frequently in recent years. An analysis of TSS over the period 1985–1991 in England and Wales revealed 103 menstrually associated cases, 26 associated with septicemia, 43 associated with localised skin infections or burns, 29 cases occurring post-operatively and 11 associated with pneumonia. Correlation was observed between the toxin and enzymes normally produced by \textit{S. aureus}. For example, strains of \textit{S. epidermidis}, \textit{S. haemolyticus} and \textit{S. intermedius} produce a haemolysin similar to the \(\delta\)-haemolysin of \textit{S. aureus}. The haemolysin is a protein of \(\geq 100\) kDa with an iso-electric point of 4.25 and is neutralised by specific antiserum and egg yolk lecithin but not by heating to 60°C. In addition to its haemolytic activity, the toxin was also shown to have some activity against tissue culture cells. Correlation was observed between haemolysis of human erythrocytes and leakage of a radioactive uridine marker from lung fibroblasts. Cytotoxic-positive strains were recognised by their ability to damage the underlying tissue culture cells. Good correlation was found between haemolytic activity and cell cytotoxicity. Whether such exoproducts play a part in pathogenesis of infection is still unresolved, although it is clear that such toxins are produced \textit{in vivo} because antibodies can be recognised in serum from convalescent patients. The incidence of toxin and aggressin production amongst clinical isolates of coagulase-negative staphylococci is summarised in table VII.  

Within the last 10 years there have also been advances in understanding of the predilection of coagulase-negative staphylococci for plastic surfaces. Initially, an adhesin is involved followed by the biosynthesis of variable amounts of extracellular slime substance (ESS) which comprises a teichoic acid-protein complex. As well as stabilising micro-colonies of the staphylococci on plastic surfaces, ESS is thought to interfere with polymorphonuclear leucocyte function preventing their ingestion and kill-

<table>
<thead>
<tr>
<th>Table IV. Features of scalded skin syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occurs in infants and young children</td>
</tr>
<tr>
<td>Very rare in adults</td>
</tr>
<tr>
<td>Sudden onset of widespread reddening of skin</td>
</tr>
<tr>
<td>Bullae formation</td>
</tr>
<tr>
<td>Widespread loss of skin exposing dark red painful surface</td>
</tr>
<tr>
<td>Caused by cleavage of middle layers of epidermis</td>
</tr>
<tr>
<td>Aetiological agent in toxicigenic \textit{S. aureus} mainly belonging to phage group II</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table V. Features of toxic shock syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Usually seen in women during menstruation</td>
</tr>
<tr>
<td>No association with particular phage types</td>
</tr>
<tr>
<td>Causes multiple clinical symptoms</td>
</tr>
<tr>
<td>Symptoms attributable to toxic shock syndrome toxin</td>
</tr>
<tr>
<td>Convalescence is not correlated with antibody production</td>
</tr>
<tr>
<td>Endotoxin bioactivity is potentiated by toxic shock syndrome toxin</td>
</tr>
<tr>
<td>Syndrome can occur following other staphylococcal infections</td>
</tr>
</tbody>
</table>

was forthcoming and an immunological cause was proposed which included activation of T cells, monocytes and macrophages. A recent study of 16 patients with Kawasaki syndrome revealed that toxicogenic bacteria could be isolated from 13, whereas only in one of 15 control subjects were similar bacteria isolated. In particular, TSST-1-producing \textit{S. aureus} was isolated from 11 of 13 toxin-positive cultures and streptococcal pyrogenic toxins B and C from the other two. There is every likelihood that the same toxins associated with TSS and capable of acting as superantigens could be closely implicated in Kawasaki syndrome. The association of Kawasaki syndrome with marked activation of T cells and monocytes/macrophages is thought to play an important part in the pathogenesis of vascular endothelial cell injury during the acute disease.

Understanding of staphylococcal disease is still unfolding. Coagulase-negative staphylococci (CNS) previously thought to be non-pathogenic are now recognised as significant causes of infections including colonisation of CSF-shunt valves, prosthetic heart valves and intravascular catheters. It is now recognised that clinically significant strains are capable of elaborating several of the toxins and enzymes normally produced by \textit{S. aureus}. For example, strains of \textit{S. epidermidis}, \textit{S. haemolyticus} and \textit{S. intermedius} produce a haemolysin similar to the \(\delta\)-haemolysin of \textit{S. aureus}. The haemolysin is a protein of \(\geq 100\) kDa with an iso-electric point of 4.25 and is neutralised by specific antiserum and egg yolk lecithin but not by heating to 60°C. In addition to its haemolytic activity, the toxin was also shown to have some activity against tissue culture cells. Correlation was observed between haemolysis of human erythrocytes and leakage of a radioactive uridine marker from lung fibroblasts. Cytotoxic-positive strains were recognised by their ability to damage the underlying tissue culture cells. Good correlation was found between haemolytic activity and cell cytotoxicity. Whether such exoproducts play a part in pathogenesis of infection is still unresolved, although it is clear that such toxins are produced \textit{in vivo} because antibodies can be recognised in serum from convalescent patients. The incidence of toxin and aggressin production amongst clinical isolates of coagulase-negative staphylococci is summarised in table VII.

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Table VI. Features of Kawasaki syndrome

<table>
<thead>
<tr>
<th>Property measured</th>
<th>Number of strains with indicated activity isolated from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>blood/endoendocarditis abscesses and wounds (42)</td>
</tr>
<tr>
<td>α-haemolysin</td>
<td>27</td>
</tr>
<tr>
<td>β-haemolysin</td>
<td>21</td>
</tr>
<tr>
<td>DNAase</td>
<td>32</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>27</td>
</tr>
<tr>
<td>Egg yolk factor</td>
<td>5</td>
</tr>
<tr>
<td>Succinic oxidase factor</td>
<td>30</td>
</tr>
<tr>
<td>Lipase/esterase</td>
<td>38</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>31</td>
</tr>
</tbody>
</table>

Adapted from Gemmell and Roberts.87

Table VII. Toxins and enzymes produced by 118 isolates of coagulase-negative staphylococci

<table>
<thead>
<tr>
<th>Property measured</th>
<th>Number of strains with indicated activity isolated from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>blood/endoendocarditis abscesses and wounds (42)</td>
</tr>
</tbody>
</table>

L. monocytogenes was first described around 40 years before the first issue of the Journal was published, initially as a pathogen of laboratory animals and then, a short time later in 1929, it was associated with an infectious mononucleosis-type syndrome in man.109 Burn subsequently reported the infective syndromes we now associated with L. monocytogenes—neonatal infection and acute meningitis in adults. Much of the first 40 years of accumulated data on all aspects of listeriosis was summarised by Gray and Killinger in an excellent review which remains valuable today. Since 1968, our understanding of listeria has advanced in many ways including veterinary infection and pathogenesis and virulence factors; however, in the following sections I will discuss further taxonomy, typing, epidemiology, human clinical infection and antimicrobial chemotherapy.

**Taxonomy**

In 1968, there was a single species in the genus Listeria, L. monocytogenes, but three associated species of uncertain taxonomic affiliation were grouped with it—L. murrayi, L. grayi and L. denitrificans. In the 1970s, it was recognised that the various non-haemolytic bio- or serovars of L. monocytogenes isolated from environmental sources may represent further species.111 In the early 1980s, on the basis of DNA-DNA hybridisation studies, strains previously designated L. monocytogenes (sensu lato) were divided into L. monocytogenes (sensu stricto) and closely related species L. ivanovii (now containing two subspecies), L. innocua, L. welshimeri and L. seeligeri.112 Whole-cell protein electrophoresis, many biochemical tests, antimicrobial sensitivity patterns, peptidoglycan structure and lipoteichoic acids in the cell walls are similar for the five major species.113-116 However, other tests may be useful in discriminating between species, including conventional biochemical tests, haemolysis, susceptibility to the antimicrobial agent fosfomycin, animal virulence and DNA typing.117-120 The classification is especially useful medically because different species have different infective potential for man. L. monocytogenes is the main pathogen of man accounting for > 99% of all infections and also causes disease in animals;111 L. ivanovii causes infection in sheep.
accounts for < 1 in 1000 infections in man and L. seeligeri has been isolated from only a single case of human acute meningitis. L. welshimeri and L. innocua have not been associated with human infection so far. In the environment, most food isolates are mainly L. monocytogenes or L. innocua but L. ivanovii may be isolated from mutton. In human faeces and sewage, L. monocytogenes and L. innocua predominate, and in a recent study, L. ivanovii and L. seeligeri were common in soil (MacGowan, unpublished data). Hence, in the study of the epidemiology of human infection and its relation to food or other environmental reservoirs, most attention should be paid to the identification and typing of L. monocytogenes.

Typing of L. monocytogenes

Serotyping was first described by Paterson and extended and developed up to the late 1970s by Donker-Voet and Seeliger (see Seeliger and Hohn). It is based on a number of O and H antigens and Listeria spp. can be divided into more than 10 serovars. Biotyping was the only other typing method available 25 years ago but it has not stood the test of time. In the mid 1990s, the range of typing methods has increased greatly and so has their importance. Bacteriophage typing was developed in the 1970s and several molecular typing methods such as random amplified polymorphic DNA (RAPD)-PCR typing and multilocus enzyme electrophoresis have become established. In the interim, several other typing methods were tried with varying success—resistogram, endonuclease restriction pattern, monocine, plasmid and DNA probe-based typing systems. None of these has gained widespread acceptance and listeria typing laboratories require a range of typing technologies because serotyping and bacteriophage typing have problems in terms of discrimination and typability. Serotyping suffers from poor discrimination: 95% of clinical isolates of L. monocytogenes fall into four main groups and 60% of human isolates in the UK are serovar 4b. Bacteriophage typing requires a set of 25–30 lytic phages and, in the UK, 82% of serovar 4b but only 37% of serovar 1/2 are typable. Phage typing is both reproducible and discriminatory for those strains which are typable. As a significant number of strains cannot be phage typed, there is a need for a third method. RAPD-PCR typing has the potential to fill this gap as it is characterised by good typability, is easy to perform and requires minimal investment in equipment, but reproducibility is difficult, no standard primers for typing are established and it is not suitable for typing large numbers of isolates (MacGowan, unpublished data). Adequate typing methods need to be centralised in a limited number of Listeria reference laboratories across the European Union if the incidence of infection is to be monitored and clusters of cases due to L. monocytogenes are to be identified rapidly and investigated. It is hoped that, by such a process of active surveillance, the large outbreaks of infection which have been such a feature of listeriosis in the last 25 years can be prevented.

Epidemiology of human infection

There are very few totally reliable studies of the incidence of listeriosis in Europe and North America because data are usually collected by national reference laboratories and are, therefore, always incomplete even in countries where listeriosis is notifiable. The incidence of infection in the UK since 1967 is shown on table VIII. An ongoing collection of data on clinical cases in Bristol since 1983 has indicated that, when local data are compared to national reporting, around a quarter to one-third of cases are not registered centrally. However, it is clear that the incidence of infection has increased since the 1960s and there was a peak between 1986 and 1989 which was associated with consumption of a specific type of pâté. The recognition that the outbreaks of listeriosis, which had been documented since the 1950s, were associated with consumption of contaminated foodstuffs radically changed our understanding of the epidemiology of human disease. This changed understanding also extended to several other areas (table IX), such as the discovery that neonates may transmit infection to other neonates in the hospital environment (reviewed by Schlech). Since the first association of epidemic listeriosis with coleslaw contaminated on a farm, it has become clear that farming practice, food preparation and distribution are all important in contributing to outbreaks which have occurred regularly throughout the 1980s and have been associated with fresh cream, pasteurised milk, fruit, Mexican-style cheese, soft cheese and ice cream, salami or Brie cheese. Sporadic cases of infection have also been associated epidemiologically with consumption of undercooked chicken or hot dogs and individual case histories have been reported that suggest linkages to some foods. These individual cases give rise to some problems in interpretation because the incubation period for human infection may be up to 90 days and it is not possible to exclude contamination of food by the infected human patient, hence they should be treated with caution. However, bacteriological monitoring of foodstuffs and updating of advice to the general public on the risks of listeriosis are likely to be important in preventing disease in the future. The isolation rates and number of Listeria spp. found in food such as pâté and soft cheese has declined recently. In contrast, isolation rates remain unchanged in raw meats, chicken, sausages and cook-chill foods, but the numbers of bacteria per gram of food may now have declined.

Clinical aspects of human infection

In the last 25 years, the main clinical syndromes of L. monocytogenes infection have changed and expanded. In 1968, the major infections were acute
meningitis—usually in adults—and disseminated infection (granulomatosis infantiseptica) in neonates. Now a more detailed picture is available (table X) with neonatal infection being divided into an early disseminated type and late infection, usually meningitis. In adults, it is now recognised that a wide range of infections can occur in immunocompromised patients; however, bacteraemia or CNS infection remain the commonest manifestations. Within these common syndromes, all of which require laboratory diagnosis, infection related to pregnancy has declined relative to other forms of infection, especially in the last 5 years and bacteraemia, a disease of immunocompromised adults is becoming increasingly important. For example, in Sweden between 1958 and 1974, 16% of adult cases presented as bacteraemia; this had increased to 25% in a review conducted between 1968 and 1978 and was 42% in a collection of cases between 1967 and 1985. In Bristol, data collected in the last decade indicate that 63% of adult cases now present as bacteraemia (MacGowan, unpublished data). As the importance of bacteraemia has increased, the relative importance of CNS infection has declined but encephalitis and abscess formation have become recognised increasingly. In addition to the risk groups for infection known in the 1960s, renal transplantation, malignancy, steroid therapy, diabetes mellitus, collagen vascular diseases and HIV infection have been added. However, some patients have no obvious predisposing factors including 30–40% of patients with meningitis and 10% with primary bacteraemia. The mortality has remained high with 50% being reported in neonates and 44% in adults. However, poor prognostic factors for acute meningitis are now recognised, such as age > 50 years, pre-existing disease, convulsions, requirement for renal or inotropic support, coma, focal neurological signs, low platelet count, raised serum creatinine and inadequate antimicrobial chemotherapy (MacGowan, unpublished data). These may help in deciding which cases need aggressive supportive therapy and optimal antimicrobial treatment.

Antimicrobial chemotherapy

The mainstays of therapy in the 1960s were penicillin, chloramphenicol and streptomycin. Sulphonamides and tetracycline were also used occasionally but it is unclear how effective they were. Ampicillin was used from 1968 onwards and was shown to be superior to penicillin in a comparative clinical trial in 1971; however, this study has been questioned and it is now thought that penicillin and ampicillin are

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**Table VIII.** The incidence of listeriosis in the UK 1967–1993

<table>
<thead>
<tr>
<th>Population</th>
<th>Period</th>
<th>Infections/10^6 population/year</th>
</tr>
</thead>
<tbody>
<tr>
<td>England and Wales</td>
<td>1967</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>1985</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>1988</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>1990–1993</td>
<td>0.22</td>
</tr>
<tr>
<td>Bristol</td>
<td>1983–1993</td>
<td>0.35</td>
</tr>
<tr>
<td>Scotland</td>
<td>1977–1986</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>1987</td>
<td>0.52</td>
</tr>
</tbody>
</table>

**Table IX.** Changes in the understanding of the epidemiology of listeriosis since 1968

<table>
<thead>
<tr>
<th>Epidemiological feature</th>
<th>1968</th>
<th>1993</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pattern of infection</td>
<td>Poorly defined—sporadic and outbreaks</td>
<td>Well defined—sporadic and outbreaks</td>
</tr>
<tr>
<td>Seasonality</td>
<td>Not known</td>
<td>Yes</td>
</tr>
<tr>
<td>Sex differences</td>
<td>Not known</td>
<td>Elderly male</td>
</tr>
<tr>
<td>Location</td>
<td>Possibly rural</td>
<td>Mainly urban</td>
</tr>
<tr>
<td>Occurrence in food</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>soil</td>
<td>Not known</td>
<td>Yes</td>
</tr>
<tr>
<td>sewage</td>
<td>Not known</td>
<td>Yes</td>
</tr>
<tr>
<td>Human carriage</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Routes of transmission</td>
<td>Proposed</td>
<td>Yes</td>
</tr>
<tr>
<td>food</td>
<td>Yes</td>
<td>Yes but rare</td>
</tr>
<tr>
<td>animal contact</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>venereal</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>fomites</td>
<td>Not known</td>
<td>Yes</td>
</tr>
</tbody>
</table>
equivalent agents.\textsuperscript{135, 156, 163, 164} Combination therapy has been common, but ampicillin and gentamicin, the most commonly used clinical combination, were shown to be synergic \textit{in vitro} only in 1972.\textsuperscript{165} Subsequently, many combinations have been tested in the laboratory\textsuperscript{166} but none has been shown to be beneficial clinically. Recently, multiresistant strains of \textit{L. monocytogenes} have been described in France,\textsuperscript{167} but such strains have not been isolated in the UK where only tetracycline resistance occurs at a frequency of about 1–2%.\textsuperscript{168} Laboratory studies have also shown that \textit{Listeria} spp. are resistant to cephalosporins but that co-trimoxazole is a bactericidal combination.\textsuperscript{169} Case reports indicate that infections respond poorly to cephalosporins but reasonably well to co-trimoxazole; however, no comparative data are available.\textsuperscript{169} Only the antimicrobial therapy of acute meningitis has been studied in any depth and the results seem to indicate that mortality can be reduced from 30% to 10–15% by the use of penicillin or ampicillin at doses of > 6 g/day.\textsuperscript{133} The addition of an aminoglycoside to ampicillin is not of proven clinical benefit,\textsuperscript{150, 158, 170} and runs the well known risks of aminoglycoside toxicity. However, ampicillin concentrations in the CSF would not exceed the MBC for \textit{L. monocytogenes} measured in the laboratory even when given in large doses, indicating that further studies are required.\textsuperscript{171} Chloramphenicol has now fallen out of favour as it has been associated with high relapse and mortality rates and the need to change therapy to alternative agents during therapy.\textsuperscript{155, 170, 172, 173} In contrast, a comparative study of chloramphenicol plus gentamicin and ampicillin plus gentamicin in the treatment of meningitis showed both treatments to be equivalent, but numbers were small.\textsuperscript{174} While it is difficult to be dogmatic, most patients with listeriosis should be treated with adequate doses of ampicillin, perhaps with an added aminoglycoside depending on the clinical status. Patients allergic to penicillins can be treated with glycopeptides for non-CNS infections or co-trimoxazole for infection in any site. Chloramphenicol plus an aminoglycoside may be of use in treating penicillin-allergic patients with acute meningitis.

\textbf{The future}

Prevention of listeriosis remains preferable to attempted cure as mortality is still considerable. This can be achieved by continued attention to food preparation, distribution and storage practices, periodic surveys of food-stuffs to identify foods of especial risk, and subsequent re-enforcement of advice on risk reduction such as adequate cooking of raw meats and avoidance of some foods. This approach should be combined with central epidemiological monitoring of the infection with supporting typing methods for early detection of case clusters and prompt investigation of potential outbreaks. Once patients have contracted infection there is evidence that treatment with large doses of penicillin or ampicillin as mono-therapy may reduce mortality.

\section*{References}


