The usefulness of biotyping in studying the epidemiology and phylogeny of salmonellae

RUTH M. BARKER and D. C. OLD

Department of Medical Microbiology, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY

Introduction

In a recent review, Le Minor (1988) classified the epidemiological markers available for the typing of Salmonella species as major or minor. Major markers were considered to be those determined by chromosomal genes and unaffected by extrachromosomal elements; minor markers were considered to be those determined by extrachromosomal elements or of insufficiently proven genetic stability. Biotyping was included in the latter category. However, our experience has suggested that biotyping affords excellent strain discrimination and that its markers are remarkably stable and generally not determined by plasmids. Accordingly, we considered it might be useful to review some of our own studies on the different ways whereby biotyping can be used to glean information about salmonellae.

Biotyping methodology

If biotyping procedures for the differentiation of strains of a species (or serotype) are to be helpful, they should satisfy several criteria. Tests should provide high discriminating ability and, ideally, epidemiologically unrelated strains should be grouped into approximately equal numbers of positive and negative types with regard to any biotyping marker chosen for inclusion in a biotyping scheme. Experience has shown, however, that a test which discriminates, say, only 5% of a minority type may still prove useful for strain recognition, as was shown by Crichton and Old (1982) in their biotyping study of strains of Escherichia coli. The results of biotyping tests should be stable and reproducible and most of the strains examined should be typable; again, for routine application, low cost and ease of performance are further desirable attributes of a good biotyping scheme.

These criteria were met in the 15 biochemical tests incorporated in the scheme of Duguid et al. (1975), devised in the first place for the biotyping of S. typhimurium. In that two-tier system, 32 potential primary biotypes were defined by the possible combinations of positive and negative reactions in the five tests that gave most discrimination of strains of S. typhimurium: tests with the substrates D-xylose, meso-inositol, L-rhamnose, d-tartrate and meso-tartrate (table I). Subtypes within these primary biotypes were defined by the reactions in ten additional biotyping tests. Full biotypes were designated by primary biotype numbers with appended letters indicating results in secondary tests, e.g., biotype designation la indicated that the culture gave positive reactions in all of the five primary and ten secondary tests of the scheme. The appendage of letter(s) other than "a" indicated negative reaction(s) in the test(s) specified by the letters b–j, x–z. The scheme has been fully described elsewhere (Duguid et al., 1975) but, for details, see footnote to table I.

This basic scheme has been applied successfully not only to the differentiation of strains of S. typhimurium, but also to strains of salmonellae of other serotypes of serogroup O4 (formerly B) and, with modifications, to serotypes of serogroups other than O4. Some examples will be described illustrating the usefulness of this biotyping scheme.

Biotyping used alone

Biotyping may be used alone in epidemiological investigations to identify strains within a serotype for which phage typing, plasmid analysis or other diverse methods of strain differentiation are not available.

At a time when infections caused by S. montevideo were common among sheep in Scotland and in the human population in England and Wales (Reilly et al., 1985), the only reported method for discrimi-
nation of strains of this serotype, a member of serogroup O7 (formerly C\textsubscript{1}), was phage typing (Vieu et al., 1981). That method, however, was not available in the UK. Therefore, attempts were made to biotype \textit{S. montevideo} by a modification of the scheme of Duguid et al. (1975), supplemented with secondary tests for the fermentation of dulcitol and \textit{L}-fucose. Biotyping identified 27 biotypes among 622 cultures of \textit{S. montevideo} (Old et al., 1985) and revealed the existence of two major biogroups, 2d and 10di, different mainly in their ability to ferment inositol (Table I).

A corresponding difference in the epidemiology of \textit{S. montevideo} was revealed from the distribution of biogroups among isolates from different countries (Reilly et al., 1985). In Scotland, biogroup 10di was the only type isolated from sheep and was also the predominant type found in other animals including man, in whom only 24\% of infections were caused by biogroup 2d. In England and Wales, on the other hand, whilst biogroup 10di was again predominant in sheep, biogroup 2d was responsible for almost all infections in cattle, poultry and man (Reilly et al., 1985). The identification of particular “sheep” strains in the UK was not correlated with severity of disease, and it may be that further progress in unravelling the epidemiology of \textit{S. montevideo} infection will be made only when biotyping is used.

### Table I. Biotype reactions of, and numbers of cultures of \textit{S. montevideo}, \textit{S. typhimurium}, \textit{S. agona} and \textit{S. paratyphi B} in, primary biotypes 1–32

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\*Character determined by reaction with D-xylose in Bitter's medium (Xyl); \textit{meso}-inositol in peptone water (Inl); \textit{L}-rhamnose in peptone water (Rha); \textit{d}-tartrate in peptone water (dTa); \textit{m}-tartrate in the place-inhibition test (mTa) (Alfredsson et al., 1972; Duguid et al., 1975).

†Cultures of a further two primary biotypes have been identified by Dr W. Rabsch (personal communication).

‡A long-term carrier yielded cultures of primary biotype 7 as well as cultures of biotypes 23 and 31 (Barker et al., 1988).
in conjunction with other schemes, such as phage typing when, and if, they become available. Thus, biotyping used alone clarified some aspects of the epidemiology of *S. montevideo* infection in the UK but the scheme remains untested in other countries where different epidemiological circumstances may exist.

**Biotyping used with another established typing system**

*S. typhimurium*. This is an ubiquitous serotype the origin of which is probably sufficiently ancient for it to have had time to diversify by mutation in chromosomal genes and by the acquisition of loss of phages and plasmids into the many subtypes now in existence (Barker, 1986). For initial epidemiological purposes, one typing method, such as phage typing (Anderson *et al.*, 1977) which discriminates 232 phage types, will suffice to indicate likely sources of infection and to highlight the major epidemic strains present in a community or a country. However, genetic variation in the typing character of epidemic strains may occur in the course of their epidemic spread. Isolates of a strain that have undergone variation in that character may be thought wrongly to belong to different clones, and isolates from different strains, happening to share the same typing character, may be thought to belong to the same clone. In such cases, multiple typing may be required to determine the true relationships among different isolates. To obtain further discrimination of phage-typed strains, we strongly recommend biotyping.

Biotyping has identified representatives of 24 primary and 187 full biotypes among >4700 strains of *S. typhimurium* examined (Anderson *et al.*, 1978; Barker and Old, 1979, 1980, 1981; Barker *et al.*, 1980; Ishiguro and Sato, 1981; Khakhria *et al.*, 1983; see table I). Thus, the potential discriminating power of biotyping, even when used alone, is high. Its value was greatly increased when used with phage typing, the combined approach distinguishing 574 different “phage-biotypes” among >2000 strains examined (Anderson *et al.*, 1978). Many of the phage types found most frequently among isolates in the UK, e.g., phage types 10, 12, 141, 170 and 193 (Communicable Disease Surveillance Centre Report, 1986; Palmer and Rowe, 1986; Barker *et al.*, 1987; Sharp, 1988) contained strains of more than one primary biotype (table II); the likely explanation of that finding is that these phage types are heterogeneous, containing different biotype lines of *S. typhimurium* that have acquired the same phage type-determining character(s).

A well-documented example of this problem has been described for strains of phage type 141, an uncommon phage type in the UK before 1972.

**Table II. Biotypes of 1667 cultures of *S. typhimurium* of 14 phage types frequently found among isolates in the UK**

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*Full biotypes represented by >3 cultures among the 4749 cultures of *S. typhimurium* (see table I). Subtype characters: results for all tests positive; a: non-fimbriate; b: non-motile; c: lactate non-fermenting; d: D-xylose non-fermenting in peptone water; e: trehalose non-fermenting; f: glycerol non-fermenting; g: L-rhamnose non-fermenting; h: inositol non-fermenting at 25°C; i: gas non-producing; j: nicotinamide-requiring; k: cysteine-requiring. y (see Duguid *et al.*, 1975).

†Cultures reported by Barker and Old (1980) have been revised with regard to reactions with D-xylose (subtype e).
Whilst it would seem reasonable to conclude that the appearance of a previously rare phage type such as 141 represented the emergence of only one kind of strain, biotyping of these phage-typed strains revealed a complex situation and identified three independent phage type/biotype groups: 141/1f, 141/9f and 141/31bde (Anderson et al., 1978; Barker and Old, 1979; Barker et al., 1980). Strains of biotypes 1f and 9f differed from each other only in the ability of the former to ferment inositol; nevertheless, that biotype property was stable and cultures from infected persons or animals in sporadic incidents or outbreaks always belonged to one or other biotype and never to both. Types 141/1f and 141/9f together accounted for almost all of the human and bovine infections by type 141 strains in the UK between 1972 and 1977, the former usually associated with human infections and the latter with incidents in cattle; type 141/31bde, the chicken type, was encountered only infrequently in the UK during that same period (Barker et al., 1980). Phage type 141 strains were relatively uncommon in the UK from 1977 to 1983, but with their reappearance in 1984, they became again the second most common phage type of *S. typhimurium* responsible for infection in man in England and Wales. Most of the animal incidents were poultry-associated, and eggs and egg products seemed to be the major sources of human infection (Communicable Disease Surveillance Centre Report, 1986). Types 141/1f and 141/9f were not involved in this most recent outbreak, a variant (141/31beg) of the poultry line being implicated for the first time in large-scale outbreaks in man or animals in the UK (Old and Barker, 1989).

On the other hand, epidemic strains may diversify in phage type by acquisition of different phage type-determining characters. In these cases, because their biotypes are unchanged, biotyping is helpful not only in confirming the relationships between strains from interconvertible phage types but also in predicting other previously unsuspected phage-type interconversions. For example, strains of the related phage types 193 and 204 accounted for most of the human and animal infections in the UK starting about 1977. Variant types of phage type 204, namely 204a and 204c, emerged thereafter and the latter quickly became the predominant type in British calves. (Wray et al., 1987). The derivation of phage type 204 strains from an ancestral strain of phage type 49 by plasmid acquisition, and the generation of the variant 204a and 204c types from phage type 204 by further reassortments of plasmid and phage DNA, have been established (Threlfall et al., 1978, 1986). These interconversions are in agreement with the findings that cultures of all four phage types belong to primary biotype 26 (Anderson et al., 1978; Barker and Old, 1980; Table II). Variant biotype lines unable to ferment xylose (biotype 26e) and inositol at 25°C (biotype 26ei) have been identified (Old, 1972; Wray et al., 1987) among cultures of the interconvertible phage types 49, 204, 204a and 204c (Table II). It remains to be resolved, however, whether the new phage type/biotype lines have evolved through multiple changes in phage type in each of the biotype lines, or by the establishment of distinct lines in each of the four phage types. Plasmid analysis has also demonstrated the interconversion of phage types 204 and 193 (Threlfall et al., 1978), the interconversion occurring probably in lines of biotypes 26a or 26e (Table II). Understanding phage-type conversions is more difficult with a phage type such as 193, the members of which are so diverse in their primary biotypes (Barker and Old, 1980; Table II). Nevertheless, biotype studies have indicated other likely phage-type interconversions, e.g., between types 193 and 141, and types 193 and 56 (Barker and Old, 1980). However, primary biotypes 1a, 2a and 3a are found in association with so many different phage types (Anderson et al., 1978) that the lines of descent of strains of phage type 193 of these biotypes will be difficult to predict.

Further examples of phage-type interconversions predicted from either combined phage type-biotype studies or from transduction studies with strains of biotype 25x (Barker and Yousuf, 1985) or with strains of biotypes 29-32 (Old and Duguid, 1979) have been discussed elsewhere (Barker, 1986).

Conversely, the conjoint use of phage typing and biotyping can also reveal the emergence of biotype variants from an epidemic strain which remains unchanged in phage type. For example, an f-tartrate non-fermenting line of biotype 26a that emerged in the UK in 1965 was recognised as a derivative of the well-established epidemic strain of phage-type/biotype 29/26a by its similarity in phage type and antibiotic resistance patterns (Duguid et al., 1975). A problem in tracing the descent of a line of phage type/biotype 44/26i from a parent line of type 44/26a by mutation in its inositol character, rather than from a different strain of phage type/biotype 44/26i by alteration in phage type, was resolved by resistotyping (Yousuf, 1984).

Modern molecular techniques, such as plasmid-profile and restriction-enzyme fragment analyses, that have been introduced recently for the epidemiological study of *S. typhimurium* (Brunner et al., 1983; Platt et al., 1986) may be as specific as the more traditional methods in establishing whether
epidemiologically related isolates belong to the same or different strains or clones (Holmberg et al., 1984). Indeed, we have found it necessary occasionally to apply both traditional and modern typing methods in a multiple typing approach to unravel particular epidemiological problems—in establishing, for example, the likely sequence of events involved when a child yielded, over a 9-month period, isolates of three lines of *S. typhimurium* distinct in phage type, antibiogram and plasmid profile (Platt et al., 1987).

*S. agona*. The origin of this serotype may not be as recent as implied by the date of its first description (Guinée et al., 1961). Nevertheless, it was rarely isolated before 1969. Thereafter, it was distributed worldwide in Peruvian fishmeal used to prepare animal feeds and quickly became established as an important serotype causing human and animal infections in many countries between 1972 and 1979 (Turnbull, 1979). Its sudden appearance and disappearance as a serotype of significance are in marked contrast to the omnipresent *S. typhimurium*.

Although some degree of differentiation was achieved by phage typing (Tyc, 1980), it was not surprising to find that the majority of biotyped cultures from diverse countries belonged to biotype 1a (Barker et al., 1982; table I). Mutational events in the epidemic strain led to the isolation of variants of biotype 1, each represented by only one or a few cultures. Among these subtype variants was included a series of isolates that fermented maltose late and a d-tartrate non-fermenting line of biotype 3a. A mutant line of rhamnose non-fermenting cultures of biotype 5a that emerged in Zaire in 1979 had become the predominant type there by 1980. Thus, even when applied to serotypes of *Salmonella* that have emerged from obscurity only recently, biotyping may afford some degree of strain discrimination. Future monitoring of strains of *S. agona* may also indicate the frequency of occurrence of new biotype variant lines and the time scale required for their establishment as epidemic strains.

**Biotyping and phylogeny**

*S. typhimurium*. Whilst multiple typing demonstrates adequately the different types of strains of a serotype present at any time in a community or country, it is insufficient to demonstrate clonal disseminations that have occurred over long periods. Stringent proof of genetic homology is required to establish which of the many types of strains around today have descended from common ancestor(s). Nevertheless, it was our biotyping studies which provided the earliest clues as to how we might obtain the necessary proof of genetic homology among strains. Our first success came with strains of *S. typhimurium* of the FIRN group, so called because they were fimbriae-, inositol- and rhamnose-negative. FIRN strains comprised 13% of the original series studied, belonged to primary biotypes 29, 30, 31 and 32, were also later shown to be Bitter’s xylose negative (table I), had been disseminated worldwide, and were diverse not only in full biotype but also in source, phage type and other characters (Duguid et al., 1975). That diversity notwithstanding, genetic evidence that FIRN strains probably had a common evolutionary origin came from experiments which showed that we never obtained fimbriate, or inositol-fermenting or rhamnose-fermenting recombinants from any FIRN × FIRN transductions (Morgenroth and Duguid, 1968; Old and Duguid, 1979; Old et al., 1980). Taken together, that body of genetic evidence implied overwhelmingly that FIRN strains were identical in the sites of their mutations in the genes for fimbriation, inositol and rhamnose fermentation, supporting the idea that FIRN strains had descended from the same ancestral bacterium which, as it spread, diversified in other characters, i.e., FIRN strains were members of an extended, and successful, clone (Anderson et al., 1978; Old and Duguid, 1979; Old, 1984).

Further biochemical and genetic studies on the xylose character (Old and Mortlock, 1979), on the inositol character (Old et al., 1980) and the meso-tartrate character (May and Old, 1980) allowed us to propose a phylogenetic tree which indicates the relationships among the four mainstream primary biotypes (1, 17, 25 and 29) and routes whereby other major biotypes probably arose as offshoots from these mainstream biotypes. In that plan, we argued that the putative archetypal bacterium could have belonged to biotype 1a, in which the genes of all 15 biotyping characters are in unmutated forms. The interested reader is directed to a recent review on the phylogeny of strains of *S. typhimurium* for more detailed information (Old, 1984).

The findings that strains of *S. typhimurium* possess a ribosomal RNA not shown by other serotypes of *Salmonella* (Smith et al., 1988), that the outer membrane-protein profile of the serotype is homogeneous (Helmut et al., 1985) and that the 17 genotypes recognised by enzyme electrophoresis cluster in one group (Beltran et al., 1988) provide further evidence that strains of *S. typhimurium*, whilst showing considerable diversity, constitute a monophyletic group.

*S. paratyphi B*. Strains defined antigenically as *S. paratyphi B* have probably been in existence for a
long time. Among them two broad categories have long been recognised: (1) strains which produce a typhoid-like illness, form a slime wall, are d-tartrate non-fermenting, have diphosph flagellar antigens and are isolated from animals infrequently, i.e., the "classical" strains of S. paratyphi B (Kauffmann, 1955); (2) strains which cause mild enteritis, do not form a slime wall, are d-tartrate fermenting and are often recovered from animals and food (Kristensen and Kauffmann, 1937); whilst most strains have diphosph flagellar antigens, some are monophasic (groups 2a and 2b, respectively), i.e., the "non-classical" strains of S. java (Kauffmann, 1955).

Biotyping of phage-typed strains provides additional discrimination and demonstrates that d-tartrate-positivity is only one of many variable biotype properties (Barker et al., 1988). Accordingly, we concur with Le Minor et al. (1982) that strains designated S. java should be looked upon as d-tartrate-fermenting variants of S. paratyphi B and that the name S. java should be discontinued. Whilst phage typing gave good differentiation of epidemic strains, it was not reliable for distinguishing "classical" from "non-classical" types because most phage types, other than Workspop, do not give a specific d-tartrate reaction (Vieu et al., 1988). Again, the d-tartrate reaction, when determined by the tests recommended by Barker (1985), did not separate "classical" from "non-classical" strains with cultures of both groups represented among biotypes 2, 3 11 and 23 (Barker et al., 1988). A prediction of the group to which a strain belongs, however, may be obtained by examination of both phage type and biotype, e.g., a culture of phage type Taunton and biotype 11 isolated from the blood of a septicaemic patient is more likely to be a "classical" type than one of phage type 1 and biotype 7 recovered from human faeces.

As well as showing diversity in phage type and biotype, S. paratyphi B strains are heterogeneous in two other characters: (i) the structure of their rRNA, with some strains giving a "coli"-type of rRNA pattern, others a "typhimurium"-type of pattern (see Smith et al., 1988); (ii) the possession of specific outer-membrane protein (omp) receptors for colicin M ("coli" type) or for bacteriophage ES18 ("typhimurium" type) (see Barker et al., 1988). On the basis of omp receptors, rRNA type, phage type and biotype, Barker et al. (1988) recognised three groups of S. paratyphi B in broad agreement with groups 1, 2a and 2b (vide supra). Thus, S. paratyphi B strains corresponding to ("classical") group 1 had a "typhimurium"-type of rRNA pattern and "coli"-type omp receptor. Although strains corresponding to ("non-classical") group 2 had the "coli"-type of rRNA pattern, they showed considerable diversity in other characters. Diphasic strains with a "coli"-type of omp receptor were found in biotypes 3, 7, 11, 23 and 31; monophasic strains with a "typhimurium"-type of omp receptor belonged to biotypes 9, 10, 12 and 26 (Barker et al., 1988). Thus, the definitive test at present available for designating strains as "classical" or "non-classical" is examination for rRNA type.

Such a degree of heterogeneity among strains of S. paratyphi B can best be explained by postulating that extant strains meeting the antigenic definition of this serotype do not necessarily have a common ancestor, i.e., they have probably descended from several archetypal bacteria, which had very different characters (Barker et al., 1988). Analysis by multilocus-enzyme electrophoresis has identified four serotypes of Salmonella—S. dublin, S. enteritidis, S. infantis and S. newport—in which isolates exhibit widely divergent clonal lineages, probably as a consequence of evolutionary convergence of antigenic phenotype by horizontal transfer and recombination of chromosomal genes mediating expression of surface antigens (Beltran et al., 1988). That kind of analysis should help to clarify the phylogeny of S. paratyphi B, strains of which seem to constitute a polyphyletic group.

Conclusions

Biotyping is an easily performed technique that can be practised without a need for expensive equipment. The substrates used in the tests defining primary biotypes, and several of those included to define secondary types, in the scheme of Duguid et al. (1975) were known to differentiate serotypes of Salmonella and, in many instances, to discriminate between strains within a serotype (Kauffmann, 1966). The same substrates and tests may well prove applicable to biotyping strains of many more serotypes than we have thus far examined. With strict adherence to the criteria for the tests as specified by Duguid et al. (1975), we achieved a considerable degree of differentiation of S. typhimurium and S. paratyphi B and some subdivision of S. agona and S. montevideo; the system has also proved helpful for biotyping strains of several other serotypes of Salmonella of serogroup O4 (unpublished observations), indicating that the 15 tests selected for the scheme detect a wide range of variant strains. When little differentiation of a serotype or of a group of isolates within a serotype was obtained by the scheme, other substrates could be included, e.g., dulcitol and fucose for S.
BIOTYPING OF SALMONELLA

monovideo. Other biochemical and nutritional tests of potential value as markers for differentiating epidemic strains of salmonellae have been described by Deschamps et al. (1982).

The obvious application of biotyping to salmonellae is in epidemiological investigations, either alone for a serotype for which no other typing method is available or together with an already established typing method, such as phage typing (Anderson et al., 1978; Barker et al., 1980) or plasmid analysis (Khakhria et al., 1983), to achieve enhanced discrimination. The findings should assist not only in interpreting the epidemiology of particular outbreaks but also in following the emergence and disappearance of particular epidemic clones. In the longer term, however, the method is likely to make an even greater contribution when applied to the selection of strains for detailed analysis by more complex and expensive methods. Such selection is required when choosing cultures for an investigation into phylogenetic relationships between strains within a serotype and between serotypes within the genus Salmonella and in choosing strains with which to work when studying, for example, factors relating to virulence. Throughout our studies, the biotype characters proved remarkably stable not only for isolates of epidemic strains in the field but also for reference cultures re-examined after storage in the laboratory for many years. Again, the 15 characters in the scheme of Duguid et al. (1975) are probably determined by chromosomal genes and, hence, not affected by plasmid carriage. In conclusion, therefore, we consider that in the typing of salmonellae, biotyping characters are markers of major importance.

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