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Gentamicin-resistant *Salmonella typhimurium* phage type 204c: molecular studies and strain diversity in a putative bovine outbreak

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SUMMARY

As part of the investigation of a putative bovine outbreak, 13 isolates of *Salmonella typhimurium* phage type 204c were subjected to plasmid analysis. Plasmid profiles suggested that several distinct strains were involved and these observations were supported by minor variations in antibiotic resistance pattern. Restriction enzyme fingerprinting and conjugational segregation of the plasmids confirmed these findings. Although 12 of the 13 isolates were resistant to gentamicin, resistance was conferred by 4 distinct plasmids; 3 of these belonged to Inc I and were distantly related on the basis of restriction fingerprints and the fourth was a resistant derivative of the 60 MDa *S. typhimurium* serotype-specific plasmid. The molecular evidence refuted the hypothesis that geographical and temporal clustering of these gentamicin-resistant isolates could be explained on the basis of a single epidmiological episode.

INTRODUCTION

Salmonella typhimurium phage type 204c was first recognized in March 1979. Although initially from bovine sources with a predominance of isolates from calves, the first human cases were seen in May of the same year [1]. Phage type 204c was characteristically antibiotic resistant from the outset and since that time strains have acquired resistance to progressively more antimicrobial agents. Gentamicin resistance was reported in 1983 [2] and later shown to be plasmid mediated [3]. The plasmids responsible for gentamicin resistance all belonged to incompatibility group (Inc) I and specified aminoglycoside 3-*N*-acetyltransferase IV (AAC(3)IV) which additionally conferred resistance to apramycin. However, molecular variation was evident among the plasmids on the basis of *Eco*R I restriction endonuclease fragmentation patterns (REFP) [4].

In Scotland gentamicin resistant (Gm^R) *S. typhimurium* was uncommon but during the latter part (September–October) of 1985 the isolation of 12 Gm^R204c from farms south-west of Glasgow suggested the incursion of a new strain on

epidemiological grounds. Although spatial clustering was evident, two of the farms that provided isolates were geographically peripheral and molecular investigation was initially undertaken to confirm the suspicion of epidemiological relatedness of the isolates. However, preliminary findings did not support the hypothesis of a defined episode and prompted a more detailed study. Past studies that have employed molecular methods have often emphasized relationships between strains from distinct outbreaks [4–6]. This report addresses not only the application of molecular analysis within a putative outbreak, but also illustrates some interpretational problems associated with the recognition of strains as related but nevertheless distinct.

MATERIALS AND METHODS

Bacterial strains and design of the investigation

Thirteen isolates previously identified as *S. typhimurium* phage type 204c were studied. Serotyping and phage typing were carried out by the Scottish Salmonella Reference Laboratory (Stobhill Hospital). All were isolated from calves between September and October 1985, originated from eight farms south-west of Glasgow, and were thought to constitute a single epizootic incident on the basis of the geographical and temporal isolation pattern, phage type and the additional observation that 12 of the 13 isolates were resistant to gentamicin-apramycin. Although the additional strain was sensitive to gentamicin it was isolated concurrently with two gentamicin-resistant strains from the same farm. Initial characterization comprised the determination of antibiogram [7] plasmid profile and restriction enzyme fingerprints [8, 9].

Thereafter, isolates identical on the basis of these parameters were assigned to 1 of 5 groups for the purposes of this report and a single representative of each group was investigated in detail as described below, except where the results obtained were unexpected and in these instances further isolates within the group were examined to clarify the anomaly.

Plasmid analysis

Plasmid profiles and restriction fingerprints were determined as previously described [9].

Plasmid DNA was initially examined in crude lysates; the equivalent of 12–20 colonies of an overnight culture on nutrient agar (Oxoid, CM3) were suspended in 300 μl of electrophoresis buffer (EB) (89 mM Tris, 89 mM boric acid, 1·25 mM-EDTA, pH 8·2) in 1·5 ml Eppendorf tubes. Two hundred microlitres of sodium dodecyl sulphate (SDS) (10% w.v in EB) was added and the tubes mixed gently by inversion. After heating at 50 °C for 5 min, the crude lysates were centrifuged for 15 min (9·980 ***g***) in a micro-centrifuge. One hundred microlitres of supernate was loaded onto a vertical agarose gel (0·7%, Sigma, type II) together with 5 μl of tracking dye (25% sucrose, 8 mM sodium acetate, 3·5 mM-SDS and 0·7 mM bromophenol blue). Electrophoresis was for 1 h at 100 V followed by 4 h at 200 V (constant voltage). Gels were stained with ethidium bromide (6 μg/ml) for 15 min, viewed using a u.v. transilluminator (365 mm) and photographed on type 665 film (Polaroid). The molecular weight of plasmids was determined by reference to

plasmids of known molecular weight; Rts 1 (120 MDa), RA-1 (85 MDa), R1 (62 MDa), R702 (46 MDa) and RP4 (36 MDa). Supercoiled ladder (Gibco-BRL) was used for the molecular weight estimation of small plasmids (< 10 MDa). Molecular weight values incorporated into plasmid profiles were determined on a minimum of two occasions.

For REFP analysis plasmid DNA was extracted after alkaline SDS lysis and purified by phenol-chloroform treatment followed by isopropanol precipitation, RNAase treatment and ethanol precipitation [9]. Restriction enzymes were obtained from Gibco-BRL (Paisley), except *Bsp* 1286 (New England Biolabs, Bishops Stratford), and used according to the manufacturer's instructions. Restriction fingerprints were compared using the Dice coefficient of similarity [10] calculated from the formula $S_D(\%) = [2m/(a+b)] \times 100$, where m was the number of restriction fragments common to two plasmids (A and B); a and b were the total number of fragments generated from each plasmid after digestion by the same restriction enzyme.

Interpretation of restriction fingerprints

The following general rules were applied to the interpretation of plasmid fingerprints.

(1) To establish that an observed fingerprint represented a variant of the reference *S. typhimurium* serotype-specific plasmid (SSP) pOG660 [9] demanded that its recognition was initially in a strain of *S. typhimurium* in which it was the sole plasmid or was similarly present in an *Escherichia coli* transconjugant. In the latter situation each of the fragments detected must have been present in the original isolate of *S. typhimurium*.

(2) If the difference between the observed variant fingerprint and pOG660 was solely due to an additional fragment or fragments, the same result must have been obtained when the plasmid was digested with twice the standard amount of restriction enzyme to exclude the presence of the products of partial digestion.

(3) Subsequent presumptive recognition of variants in clinical or veterinary isolates that contained additional plasmids was accepted if the additional plasmid was substantially different in copy number [11] or if the variant had lost at least one fragment.

Analysis of transconjugants

Single colonies were removed from each of the selection plates, the number of which was determined by the resistance markers present in the donor strain. Confirmation of plasmid transfer was as previously described [12]. Initially six colonies were tested. However, where co-transfer of multiple plasmids occurred or where transconjugant plasmids did not correspond to those present in the donor, further colonies were examined. REFP analysis of transconjugants was carried out in parallel with donor isolates after digestion with the same restriction enzyme either on the same gel or after computerized data storage and retrieval as described below. Transconjugant plasmids were only accepted as representative of the donor isolate if there was no detectable difference in the total molecular weight from a plasmid profile or all restriction fragments were present in the donor fingerprint.

Computer-aided analysis of restriction fragments

Restriction fragment mobility in ethidium bromide-stained agarose gels was recorded on Polaroid type 665 film and input to an Apricot 286 microcomputer via a digitizer (Summagraphics). Each gel was calibrated using restriction fragments from a *Pst* I digest of bacteriophage lambda either alone or in combination with an *Sma* I or *Hind* III digest of the same phage. The molecular weight of these fragments was fitted to a robust modified hyperbola [13] from which the size of restriction fragments in adjacent tracks was estimated. Numerical values were stored for subsequent graphical output which was on a logarithmic scale.

RESULTS

Plasmid profiles and antibiograms of the 13 isolates are shown in Table 1 together with the characteristics of two variants (GRI 31586-4 and GRI 31986-4) not evident in the original cultures and presumed to be spontaneous (laboratory) derivatives. The distribution of isolates assigned to one of five groups on the basis of preliminary characterization and in relation to the farm of origin is shown in Fig. 1 which provides an overview of the putative episode and illustrates the strain diversity within an otherwise circumscribed epidemiological incident.

Variation in antimicrobial susceptibility

Three types of resistance pattern were notable among the isolates (Table 1). Those organisms assigned to Group I specified resistance to kanamycin-neomycin (Km) whereas this determinant was absent from all other groups. Isolates within Group III were designated as partially resistant to both streptomycin (Sm) and gentamicin (Gm). Disk-diffusion tests consistently showed a small clear zone of inhibition ($\leqslant 6$ mm radius compared with a mean value of 13 mm for a sensitive strain of *S. typhimurium*; S.D. = 1·5 mm) with a scattered growth within the inhibition zone. However, repeat testing either directly or with intermediate subculture of both partially resistant or apparently fully resistant clones produced identical results from which we concluded that variation in Sm/Gm resistance was a result of variable expression of these determinants. Thirdly, two isolates of identical resistance pattern (Group IV and V) were sensitive to ampicillin which is an uncommon finding among phage type 204c. They differed from each other in plasmid profile and underwent different spontaneous variation in both antibiogram and plasmid profile. The isolate GRI 31486, although sensitive to gentamicin was included in the study since it shared some resistance and molecular characteristics with GRI 31286 and GRI 31386 and was isolated concurrently with the gentamicin resistant strains from the same farm.

Resistance transfer by conjugation

More than 60 different transconjugants were obtained from standard conjugation procedures using the six donor isolates (Table 1). Many of these had acquired more than one plasmid in a variety of combinations. Twenty-eight transconjugants that acquired single plasmids were analysed in detail. From the combination of these results we concluded that the small plasmids of 5·0 MDa plasmid specified Sm/Su resistance, the 7·0 and 3·0 MDa plasmids did not confer

Table 1. *Characteristics of* S. typhimurium *DT 204c*

Designation	Resistance determinants*	Plasmid profile (MDa)	Group
GRI 30986†	Ap Km Tc Cm Sm Tp Su Gm	120:96:60	I
GRI 31086	Ap Tc Cm pSm Tp Su Gm	120:60:7·0	III
GRI 31186†	Ap Tc Cm pSm Tp Su pGm	120:60:7·0	III
GRI 31286	Ap Km Tc Cm Sm Tp Su Gm	120:96:60	I
GRI 31386	Ap Km Tc Cm Sm Tp Su Gm	120:96:60	I
GRI 31486†	Ap Km Tc Cm pSm Tp Su	120:60:36 :3·5	II
GRI 31586†	Tc Sm Tp Su pGm	85:60:5·0:3·0	IV
GRI 31586-4†	Tc Sm Su	60:5·0	IVa
GRI 31686	Ap Km Tc Cm Sm Tp Su Gm	120:96:60	I
GRI 31786	Ap Km Tc Cm Sm Tp Su Gm	120:96:60	I
GRI 31886	Ap Km Tc Cm Sm Tp Su Gm	120:96:60	I
GRI 31986†	Tc Sm Tp Su pGm	70:3·0	V
GRI 31986-4	Fully sensitive	60:3·0	Va
GRI 32086	Ap Tc Cm pSm Tp Su pGm	120:60:7·0	III
GRI 32186	Ap Tc Cm pSm Tp Su pGm	120:60:7·0	III

* Ap, ampicillin; Km, Kanamycin (neomycin); Tc, tetracycline; Cm, chloramphenicol; Sm, streptomycin; Tp, trimethoprim; Su, sulphonamide; Gm, gentamicin (tobramicin-apramycin). The prefix 'p' before an individual determinant indicates significant resistance but a detectable zone of inhibition.

† Isolates used as R-plasmid donors in conjugation.

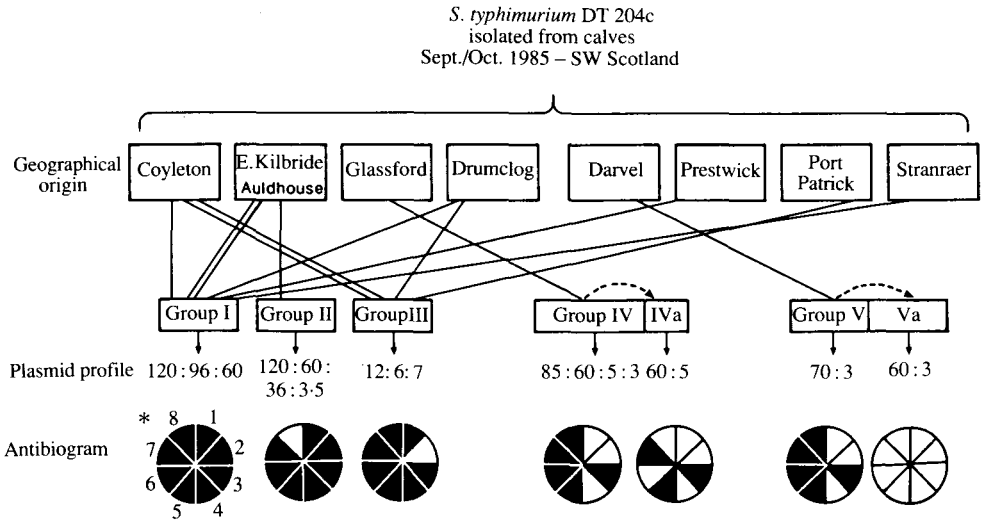

Fig. 1. Summary of the putative outbreak: * ■ denotes resistance, □ denotes sensitivity to 1, ampicillin; 2, kanamycin; 3, tetracycline; 4, chloramphenicol; 5, streptomycin; 6, trimethoprim; 7, sulphonamides; 8, gentamicin (tobramycin and apramycin). a: (in IVa and Va) denotes a laboratory derivative.

resistance to any antimicrobial agents and the analysis of transconjugants provided no clear evidence for the 3·5 MDa plasmid.

The 120 MDa plasmid specified resistance to Tc, Cm, Sm, Tp, Su. However, individual transconjugant clones from GRI 30986 (Group I) had lost the Sm/Su markers, GRI 31086 (Group II) has lost Cm and two clones from two Group III strains that harboured this plasmid alone had acquired pGm resistance.

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Table 2. *Characteristics of gentamicin resistance plasmids in* E. coli *K12 transconjugants*

Donor strain	Group	Transconjugant* resistance	Plasmid profile	R-marker selection
GRI 30986	I	Sm pGm	60	Sm
		pSm pGm Su	60	Gm
		Tc pSm pGm	60	Tc
		pSm pGm	60	Tc
GRI 31086	III	pGm	60	Gm
GRI 31186	III	pGm	60	Gm
		Ap pGm	60	Ap
GRI 31586	IV	Tc Sm pGm Tp	85	Gm
		Tc Sm pGm Tp	85	Sm
		Tc Sm pGm Tp	85	Tp
		Tc Sm pGm Tp	85	Tc
GRI 31986	V	Tc Sm pGm Tp	70	Sm
		Tc Sm pGm Tp	60	Tc

* *E. coli* K12; abbreviations as Table 1.

Gentamicin resistance plasmids

Considerable variation was seen among transconjugants that specified gentamicin resistance. This is shown in Table 2 and in general reflected the resistance determinant used to select the incoming plasmid with respect to Groups I, III and IV. Transconjugants from GRI 31586 (Group IV) did not show this variation irrespective of the selecting determinant. The variation, with the exception of Tc-selected transconjugants of GRI 31986, did not significantly alter the plasmid size as determined by agarose electrophoresis although molecular variation was evident in restriction enzyme fingerprints as described below.

Restriction enzyme fingerprinting

On the basis of REFP analysis all isolated harboured the 60 MDa *S. typhimurium* serotype-specific plasmid (≡ pSLT, MP10, pOG660). However, several harboured molecular variants of it that have previously been described in detail[9]. Among Group I isolates it was present as a cointegrate plasmid of 96 MDa; among Groups II, III and IV the *Pst* I and *Sma* I fingerprints were typical of the 60 MDa plasmid. GRI 31986 harboured this plasmid as a mobilizable R-plasmid that specified Tc, Sm, pGm, Tp determinants. The spontaneous laboratory derived strain (GRI 31986-4) lacked all resistance determinants, together with the 11 kb *Sma* I fragment of the SSP and was designated pOG676.

Gentamicin R-plasmids which ranged in molecular weight from 60 MDa (Groups I–III) to 85 MDa (Group IV) differed markedly in their restriction fingerprints (Fig. 2). Three distinct types were evident which corresponded to Groups I, III, and IV in addition to the Gm^R derivative of pOG660. Groups I and III were most closely related on the basis of a Dice coefficient of similarity (S_D = 32%) whereas Group IV was only minimally related to both other groups (S_D = 16% in both cases although the matching restriction fragments were different in each case).

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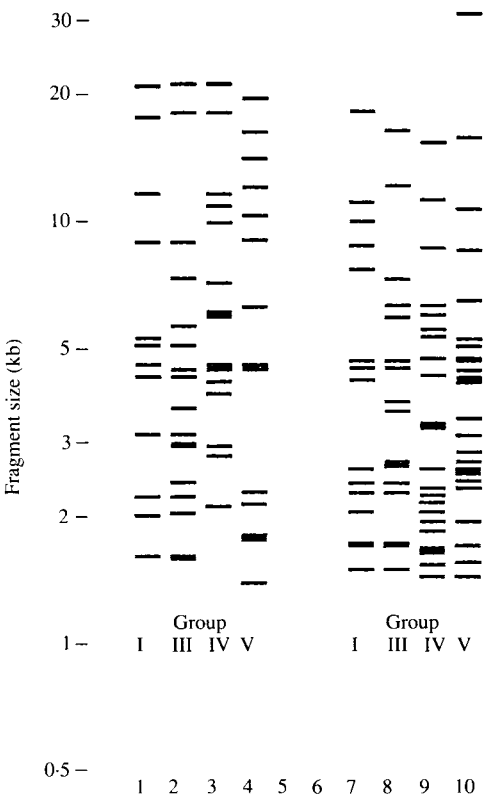

Fig. 2. *Eco*R I (tracks 1–4) and *Sma* I (tracks 5–8) restriction fingerprints of gentamicin resistance plasmids from *S. typhimurium* DT 204c.

Gentamicin R-plasmids, with the exception of the single Group IV isolate, exhibited conjugational rearrangements, some examples of which are shown in Fig. 3.

Epidemiology

Review of the available epidemiological data provided no unequivocal information beyond the observation that each of the farms concerned had purchased calves during the weeks that preceded the isolation of *S. typhimurium*. However, the following additional observations provide circumstantial evidence to corroborate the molecular evidence that the clustered isolation of these strains did not represent a single incident. Strains that belonged to Groups I and III were associated with calves purchased from one of two markets (Ayr and Lanark) or both.

Those farms from which more than one line of phage type 204c was isolated (Groups I and III) had purchased calves in more than one batch and in significant numbers; among these three farms there were 28 calf deaths, and more than 60 clinically affected. The disease pattern was consistent with exposure to infection at the time of purchase and imported into the respective farms from an external source (markets). The geographical distribution of the farms does not entirely

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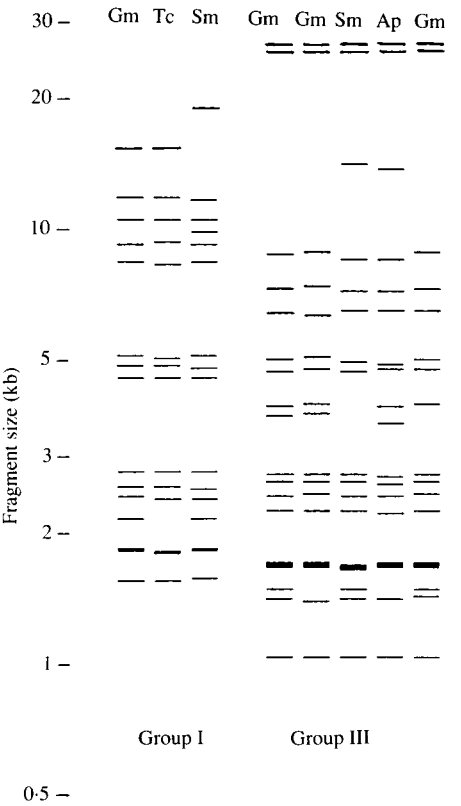

Fig. 3. *Sma* I fingerprints of conjugational rearrangements of gentamicin resistance plasmids from Group 1 (tracks 1–3) and Group III strains (tracks 4–8).

exclude the possibility of farm to farm transmission but this is thought to be improbable. No common direct or indirect contact such as staff or equipment could be identified.

DISCUSSION

The results presented illustrate that although superficially similar in terms of phage type and resistance to gentamicin-apramycin the strains of *S. typhimurium* associated with this putative episode clearly belonged to different lines of DT 204c and therefore did not represent a single incident. On the basis of the groups to which the isolates were initially assigned after preliminary investigation, Group I was unique in that the *S. typhimurium* SSP was present as a 96 MDa cointegrate plasmid [9] and the 60 MDa plasmid present was an Inc I R-plasmid that specified Gm resistance. This contrasts the usual situation in *S. typhimurium* where 60 MDa plasmids correspond to the SSP [14, 15].

The single group II isolate differed in several respects from those assigned to Group I; notably it autonomously harboured the two plasmids that gave rise to the Group I cointegrate plasmid. However, it lacked the 60 MDa plasmid that specified resistance to gentamicin and possessed an additional 3·5 MDa small plasmid. Although both Group I and II strains were isolated from the same farm it seems unlikely that the cointegration event took place *in situ* in the light of both

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the additional strain differences and the geographical distribution of the Group I isolates. It is nevertheless possible that an isolate of similar or identical characteristics to Group II represents a progenitor of Group I.

Group III isolates were distinct from Groups I and II. Although the 120 MDa plasmid was common to each of these groups there were few additional similarities; the small plasmids (Groups II and III) were distinct on the basis of *Pst* I fragmentation patterns and Group III isolates harboured two 60 MDa plasmids that co-migrated. These were the SSP (identical to the Group II 60 MDa plasmid) and a gentamicin R-plasmid only distantly related to the 60 MDa plasmid of Group I (Fig. 3).

Groups IV and V, each represented by a single isolate, differed from the other groups by the absence of the 120 MDa plasmid and Ap, Km and Cm resistance determinants. Although of identical antibiogram they differed from each other markedly in molecular characteristics; gentamicin resistance was specified by an 85 MDa Inc I plasmid (Group IV) which showed molecular stability after conjugative transfer to *E. coli* K12 in that all transconjugants had identical fingerprints, irrespective of the antimicrobial agent used for selection. This contrasted the gentamicin R-plasmids from strains assigned to Groups I and III. Gentamicin resistance in GRI 31986 (Group V) was conferred by an unrelated plasmid (pOG672) derived from the SSP of this serotype [9]. The *Sma* I fingerprint contained six fragments additional to those of the SSP, of which four were common to the 85MDa Gm R-plasmid from the Group IV isolate. The resistance determinants specified by both of these plasmids were the same and the fully sensitive laboratory derivative (GRI 31986-4) harboured an SSP that lacked one of the characteristic *Sma* I fragments. These findings suggest that pOG672 may have arisen by the acquisition of a composite transposon and the sensitive derivative by imprecise excision.

Threlfall and colleagues [4] described three gentamicin R-plasmids distinguishable on the basis of molecular characterization among strains of DT 204c isolated between 1983 and 1985. The resistance determinants specified were the same as Group I isolates reported here. Comparison of *Eco*R I fingerprints indicates that none of the gentamicin R-plasmids reported here were identical to those previously described [4]. Together with the considerable diversity of Gm R-plasmids seen within this episode and the molecular variations seen after conjugation (Fig. 3) it appears that Gm R-plasmids are subject to rapid evolutionary divergence. This situation is not restricted to Gm R-plasmids in *S. typhimurium*. Trimethoprim R-plasmids in *E. coli* [16] and ampicillin R-plasmids [17] show similar divergence. However, this contrasts the molecular conservation of SSPs of *S. typhimurium* [9], *S. enteritidis* and *S. dublin* and demonstrates that the individual plasmid components of plasmid profiles have the capacity to evolve at different rates. In turn this has clear implications for their use in epidemiology; not only can strains of disparate origin exhibit the same profile by virtue of acting as host to highly conserved plasmids, but also strains that exhibit quite distinct profiles can result from rapid divergence over a short time. Thus, in the investigation of any specific incident the interpretation of plasmid profiles ideally requires prior familiarity with the evolutionary behaviour of the plasmids common within the genus under investigation to allow distinction between the opposing situations described above

or the recognition of intermediate status. Given the availability of such baseline data the use of case-matched studies [18] in interpretation, where putative outbreak strains are compared with a similarly sized sample of unrelated isolates, may be appropriate. However, in the absence of sufficient background information the scope for bias in case-matched studies [19] argues against the general adoption of this method.

The now widespread application of plasmid profile and REFP analysis in epidemiology suggests the need to develop a coherent interpretative framework which takes account of the multiplicity of factors which differ with both molecular, bacteriological and epidemiological circumstances.

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