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Restriction Enzyme, Plasmid Profile Analysis and Antibiotic Resistance of *Salmonella* Typhimurium of Poultry Origin Isolated from Egyptian Farms

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Abstract: Ten selected isolates of *S.* Typhimurium (5 from chicken, 4 from ducks and 1 from turkey) isolated from poultry flocks in 5 geographical areas at Egypt were characterized by phenotypic and genotypic methods. None of the examined S. Typhimurium isolates was positive for infant mice assay. Meanwhile, all of the examined isolates were positive for Vero toxin activity. All *S.* Typhimurium isolates were pathogenic for one-day-old chicks with a total mortality of 56%. The antibiotic sensitivity test revealed that 100, 80, 70 and 70% of the isolates were sensitive to chloramphenicol, ciprofloxacin, enrofloxacin and norfloxacin, respectively. While 100% of the isolates were resistant to amoxicillin and ampicillin and 70% were resistant to neomycin. *S.* Typhimurium showed a degree of variation in plasmid number (from one up to six plasmids) and molecular size (from 583 up to 16732 bp). All *S.* Typhimurium isolates harbored plasmid of 11425 bp, while 40% of the isolates had plasmid of 1893 bp. Meanwhile, plasmids of 16732, 1326 and 583 bp were detected in 30% of the isolates. The largest plasmid of 16732 bp was detected in 30% of the Egyptian isolates that showed the highest incidence of antimicrobial resistance and mortality rates among chicks, which may probably, contributes to the high virulence of these isolates. Restriction endonuclease analysis (REA) of the genomic DNA revealed that 24-34 bands were identified among isolates. Ten bands were common to all investigated isolates.

Key words:

INTRODUCTION

Salmonella infection is one of the most serious problems that affect poultry industry, causing high economical losses not only due to high mortality in young birds, but also for the debilitating effect, which predisposes for many other diseases. *S.* Typhimurium has been among the most common serovars isolated from poultry in many countries and it represents more than 40% from all *Salmonella* isolates during the period from 1950 until 1970 [1]. Since the 1940s, there has been a rapid increase in the isolation of the non host specific *S.* Typhimurium serovars from animals and humans [2]. Therefore, several serovars can be transmitted from one individual to another [3]. Poultry and poultry products are consistently identified as important sources of salmonellae that cause human illness [4]. More than one-third of food-borne salmonellosis outbreaks in humans in the United States between 1983 and 1987 were associated with poultry meat or eggs [5]. The total annual costs of medical care and lost productivity resulting from food-borne *Salmonella* infections of humans in the United States have been estimated at up to $ 3.5 billions [6]. Culture-confirmed human cases of salmonellosis reported by the Disease Control and Prevention Centers (CDC) in the United States have increased steadily, rising from 26,326 in 1972 to 39,033 in 1996 [7]. According to CDC, salmonellae are responsible for an estimated 1.34 million illnesses, 16,430 hospitalizations and 582 deaths each year [8].

Molecular typing (genotyping) has a very important role in obtaining further discrimination when traditional typing (phenotype) indicates close relationships between isolates, especially when isolates are from a narrow geographical region or isolates obtained during a limited time. The interpretation of molecular typing is considered as a very important epidemiological marker [9].

Investigations into the epidemiology of animal and human salmonellosis require the use of several typing

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methods for typing the strains in to groups below the level of serovars. Therefore, this study was planned to study the plasmid profile analysis and restriction endonuclease analysis (REA) of the chromosomal DNA among *S.* Typhimurium isolated from different poultry farms in comparison to virulence attributes and antibiogram typing of the investigated strains.

MATERIALS AND METHODS

Local Isolates of Salmonellae: Ten *S.* Typhimurium isolates were isolated from broilers (5 isolates), ducks (4 isolates) and turkey (1 isolate) from different Egyptian governorates as shown in Table 1.

Colonial Morphology, Collier *et al.* [10]: A loopful of the obtained culture was inoculated into selenite-F broth and incubated at 37°C for 16 hours then streaked on to the surface of MacConkey's agar, *Salmonella-Shigella* agar XLD and Hektoen enteric agar and incubated at 37°C for 24 hours.

Microscopical Examination: The suspected *Salmonella* colonies were picked up and stained with Gram's stain method and examined microscopically.

Motility Test: Motility was assured by growing the bacteria into semisolid agar.

Biochemical Identification: The purified isolates of salmonellae were examined by different biochemical reactions according to Quinn *et al.* [11]. KB011 Hi-*Salmonella* Identification Kit was also used in biochemical identification.

Serological Identification of Salmonellae: The isolates preliminary identified biochemically as *Salmonella* were subjected to serological identification according to Kauffman [12] using diagnostic polyvalent (O, H) and monovalent *Salmonella* antisera (Difco) for serological identification of *Salmonella*.

Sensitivity Test for *Salmonella* Isolates: The sensitivity of the *Salmonella* isolates was tested towards: amoxicillin, ampicillin, chloramphenicol, ciprofloxacin, enrofloxacin, flumequine, gentamicin, neomycin, norfloxacin and oxolinic acid according to Finegold and Martin [13]. Interpretation of the results was done according to Koneman *et al.* [14].

Table 1: Source and host of different *S.* Typhimurium isolates

Isolate number	Source	Host
1	Kalubia	Duck
2	Kalubia	Broiler
3	Menofia	Duck
4	Menofia	Broiler
5	Behera	Duck
6	Behera	Broiler
7	Dekahlia	Broiler
8	Giza	Duck
9	Giza	Broiler
10	Giza	Turkey

Detection of *Salmonella* Toxins: These steps have been achieved by infant mouse assay [15]and by verocytotoxicity assay [16].

Pathogenicity of S. Typhimurium Strains in One –Day-Old Chicks [17]: Before pathogenicity test, the birds were confirmed to be *S.* Typhimurium free following cloacal swabbing. Then birds were divided into 10 groups, each of 10 birds (1-10). Group 11 consists of 10 birds, which acted as negative control. Chicks of the first 10 groups (1-10) were inoculated orally using 1-ml sterile syringe with 0.5 ml of overnight broth culture containing 10^6 colony forming unit (CFU)/chick of one isolate of S. Typhimurium. The control group was given 0.5 ml of sterile broth orally. Each experimental group was kept in separate cages to prevent any cross-contamination. The chicks were feed on conventional broiler feed without antimicrobial agents. The mortality rate was recorded after 7 days of infection.

Plasmid Profile Analysis, Sambrook *et al.* [18] and Towner and Cockayne, [19]

Extraction of *Salmonella* Genome, Scheppler *et al.* [20]: It was carried out as recommended by the manufacturer of the Genomic DNA purification kit.

Restriction Enzyme Analysis [18]: It was carried out as recommended by the manufacturer of the restriction enzyme–and buffer. DNA was digested with HindIII restriction enzyme [18] and analyzed by electrophoresis on submarine ethidium bromide containing 0.7% agarose gels.

RESULTS AND DISCUSSION

Zoonotic *Salmonella enterica* serovars are among the most important agents of food – borne infections throughout the world. The non host specific *Salmonella* serovars such as *S.* Enteritidis and *S.* Typhimurium are the agents of paratyphoid infections in domestic poultry and a major concern for food safety [21].

Table 2: Antibiotic sensitivity test of *S.* Typhimurium isolates

	Resistant		Intermediate		Sensitive	
Antibacterial agent	No.	%	No.	%	No.	%
Amoxicillin	10	100%	0	0%	0	0%
Ampicillin	10	100%	0	0%	0	0%
Chloramphenicol	0	0%	0	0%	10	100%
Ciprofloxacin	0	0%	2	20%	8	80%
Enrofloxacin	0	0%	3	30%	7	70%
Flumequin	3	30%	1	10%	6	60%
Gentamicin	4	40%	2	20%	4	40%
Neomycin	7	70%	3	30%	0	0%
Norfloxacin	0	0%	3	30%	7	70%
Oxolinic Acid	3	30%	2	20%	5	50%

No. = Number of S. Typhimurium isolates

The percentage was calculated according to the number of isolates (10)

Ten selected strains of *S.* Typhimurium (5 from chicken, 4 from ducks and 1 from turkey) isolated from poultry flocks in 5 Egyptian geographical areas (Behera, Dakahlia, Giza, Kalubia and Menofia) were characterized by phenotypic and genotypic methods to compare the usefulness of the methods in epidemiological studies.

Antimicrobial resistance in pathogenic bacteria of animal and human origin is a major public health issue. As shown in Table 2, 100, 80, 70 and 70% of the isolates were sensitive to chloramphenicol, ciprofloxacin, enrofloxacin and norfloxacin respectively. In this concern, Chaslus-Dancla and Martel [22] recommended that the fluoroquinolones are drug of choice for treatment of invasive salmonellae and some antibiotics, namely enrofloxacin, danofloxacin and marbofloxacin, are also specifically approved for therapeutic veterinary use. Furthermore, Mhand *et al.*[23] concluded that *S.* Typhimurium isolates were susceptible to chloramphenicol, tetracycline and quinolones.Thereafter, *Salmonella* species with high level of drug resistance have been continuously reported and the exact location and changes of genes associated with a mutation of these species have been searched [21].

In the present study 100% of the examined isolates were resistant to amoxicillin and ampicillin while 70% were resistant to neomycin. Most of *S.* Typhimurium strains typically carry resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (R-type), but resistance to 9 or 10 different antibiotics also occurs [24]. *S.* Typhimurium was resistant to sulfonamide, tetracycline and trimethoprim sulfamethoxazole [25]. Percentage resistance to all antimicrobials was greatest among strains of *S.* Typhimurium, *S.* Typhimurium var Copenhagen, *S.* Derby and *S.* Heidelberg, among *S.* Typhimurium and *S.* Typhimurium var Copenhagen (n=54), resistance to ampicillin, ticarcillin, sulfamethoxazole, streptomycin and tetracycline, singly, ranged between 61 and 69% [26]. This result was supported by the result of Kasimoglu Dogru *et al.* [27] who concluded that out of 32 Salmonella strains isolated from 400 chicken carcasses, 22 (68.75%) displayed multi-drug resistance. Thirty-two (100.0%) of the isolates were found to be resistant to penicillin G, 20 (62.5%) to nalidixic acid, four (12.5%) to cephalothin, two (6.2%) to streptomycin and two (6.2%) to tetracycline.

Within *Salmonella* and especially *Salmonella* enterica serotype Typhimurium, multiple-antibiotic resistant strains are isolated with increased frequency. These multiple antibiotic resistant serotype Typhimurium strains cause particular concern because of their increasing prevalence in humans [28]. Multidrug resistant *Salmonella* serovars cause severe and septicemic salmonellosis more frequently than those that are not resistant [29, 30]. The taking of antimicrobials by patients within a month of the onset of salmonellosis may aggravate the severity of disease symptoms and result in increased hospitalization rates [31]. Contributing mechanisms are thought to include selection for the resistant pathogen, concomitant killing of protective normal flora and increased colonization of the enterocytes by the offending bacteria [32].

Virulence of salmonellae means the ability to produce a pathological state in a certain growth phase, in a particular host under certain conditions. The studies on the pathogenesis of *Salmonella* revealed several virulence factors including certain bacterial structures and products [33]. Although the microbial factors that contribute to the virulence in *Salmonella* have not yet been fully elucidated, investigations on the virulence

Table 3: Virulence attributes of *S.* Typhimurium strains

					Mortality					
					1st week				Total	
Group No.	Isolate No.	Infant mice assay	Cytop-thic effect	No. of chicks/group	1st day	2nd day	3rd day	4th day	No.	%
1	1	-	++	10	1	1	1	-	3	30%
2	2	-	++	10	2	2		-	4	40%
3	3	-	+++	10	4	3	1	-	8	80%
4	4	-	+++	10	3	3	1	-	7	70%
5	5	-	++	10	2	2	1	-	5	50%
6	6	-	++	10	2	3	1	-	6	60%
7	7	-	+++	10	5	4	-	-	9	90%
8	8	-	++	10	1	2	-	-	3	30%
9	9	-	+++	10	1	3	2	-	6	60%
10	10	-	+++	10	1	2	2	-	5	50%
Total			100		22	25	9	-	56	56%
Control			10		-	-	-	-	0	0%

(++): The No. of rounded and detached cells was (50%-75%)

(+++): The No. of rounded and detached cells was (75%-90%)

characteristics of *S.* Typhimurium strains of chicken origin were conducted in the present study to better understanding of the pathogenesis of avian infection with *Salmonella* serotypes.

Table 3 reveals that none of the examined *S* Typhimurium isolates could produce heat stable enterotoxin, using infant mouse assay. This result was supported by the result of Wallis *et al.* [34] who recorded that *S*. Typhimurium extracts were inactive in infant mouse assay for enterotoxin. While [35] detected a heat labile enterotoxin in 44% of 123 S. Typhimurium strains from animal sources.

Table 3 reveals that all of the examined *S.*Typhimurium isolates produced cytopathic response for Vero cells (100%). This result was supported by the result of Koo *et al.* [33] who found that cytotoxin produced by *Salmonella* inhibited the protein synthesis in Vero cells and caused significant loss of membrane integrity of Vero cells. Furthermore, cytotoxin production has been demonstrated by O'Brien *et al.* [36] in *S.* Typhimurium strain. Infection of human macrophages with *S.* Typhimurium produced delayed cytotoxicity, cell detachment and apoptosis [37]. Meanwhile, no cytopathic effect was observed with *S.* Typhimurium tested by Jesudason *et al.* [38] in Vero cell.

Pathogenicity studies following oral inoculation of *S*. Typhimurium isolates for one-day-old chicks as shown in Table 3 suggested that all *S.* Typhimurium isolates were pathogenic for one-day-old chicks, the mortality rates were 30% (Strain No. 1 & 8), 40% (Strain No. 2), 50% (Strain No. 5 & 10), 60% (Strain No. 6 & 9), 70% (Strain No. 4), 80% (Strain No. 3) and 90% (Strain No. 7). The control group survived during the course of the experiment without any symptoms. *S.* Typhimurium isolates were re-isolated from all internal organs. Twenty four hours post-inoculation, *Salmonella* was recovered from 20, 30, 40, 40 and 100% of the liver, thymus, spleen, bursa and ceca samples respectively, after oral inoculation [39].

In the present study the highest rates of mortalities, 70, 80 and 90% were associated with isolates number 4, 3 and 7, respectively. These isolates were harbored the largest plasmid of 16732 which may probably contribute towards high virulence of these isolates in one-day-old chicks.

Bacterial strains were screened for plasmid DNA by Sambrook *et al.* [18], Towner and Cockayne [19] procedures. Most of the isolates showed a degree of variation in plasmid number (from 1 plasmid up to 6 plasmids) and molecular size (from 583 bp up to 16732 bp).

As shown in Table 4 and Photo 1, all *S.* Typhimurium isolates harbored plasmid of 11425 bp, 40% of the isolates had plasmid of 1893 bp. Meanwhile, plasmids of 16732, 1326 and 583 bp were detected in 30% of the isolates. Nakamura *et al.* [40]concluded that, when *S.* Typhimurium isolates isolated from animals reared in limited areas exhibit identical or similar plasmid patterns, they are derived from the same source and that when isolated in a

Table 4: Relationship between plasmid profile, antibiotic resistance and pathogenicity in one-day-old chicks

Isolate No.	Number of plasmids	Plasmids molecular size bp	Percentage of antibiotic resistance	Mortality percentage of one-day-old chicks
1	1	11425	30%	30%
2	2	11425	30%	40%
		1326		
3	6	16732	60%	80%
		11425		
		2413		
		1893		
		583		
		1521		
4	5	16732	60%	70%
		11425		
		5012		
		583		
		3275		
5	3	11425	20%	50%
		1893		
		5592		
6	3	11425	20%	60%
		1893		
		5592		
7	6	16732	60%	90%
		11425		
		2413		
		583		
		1893		
		2134		
8	1	11425	20%	30%
9	4	14249	40%	60%
		11425		
		1326		
		1953		
10	4	14249	30%	50%
		11425		
		1326		
		1953		

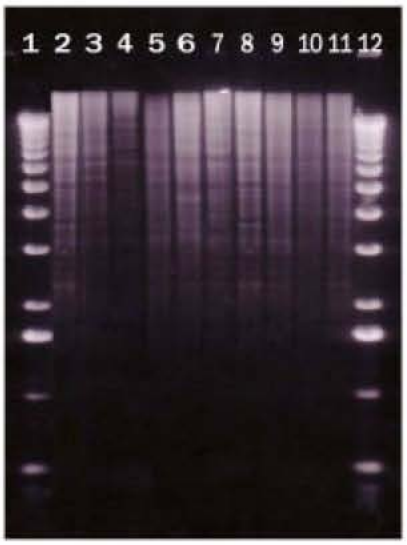
Photograph 1: The plasmid profile analysis

limited area exhibit quite a different plasmid patterns, these isolates were derived from independent sources. *S.* Typhimurium strains contained 4 plasmids of 45, 16, 6 and 3.5 MDa [41]. Thus, plasmid analysis appears to be the more effective method for grouping strains with the same serotype. Previous studies have found plasmid profiling to be of use in intra-serotype differentiation [42 - 44]. Results of plasmid profiling showed that all DT104 strains retain a 90 kb virulence plasmid, 20 of 36 strains possessed few additional small plasmids ranging from 2 to 4 kb [45]. Also, Majtan *et al*. [46] recorded that the 90 kb virulence plasmid was contained in 138 (89.6%) of *S.* Typhimurium isolates. Twenty *S.* Typhimurium isolated from poultry were examined by Bhattacharya *et al*. [47] to determine the correlation between amplification of virulence gene, presence of plasmid and virulence in mice and chicks. They concluded that there is no correlation between presence of plasmid, virulence in mice and chicks and amplification of virulence gene.

Several investigators reported that resistance to different antimicrobial agents was mediated by a large plasmid [48, 49]. In the present investigation, the largest plasmid of 16732 bp was detected in 30% of the Egyptian isolates that showed the highest incidence of

Table 5: Computer analysis of DNA HindIII Digestion of S. Typhimurium isolates

	Isolate No.									
Molecular size range (bp)	1	2	3	4	5	6	7	8	9	10
15000-15999	15119	15053	15184	-	15053	15251	15451	15384	15384	15384
14000-14999	14412	14350	14412	14538	14350	14350	14538	14665	14665	14665
13000-13999							13919	13919		13919
	13859			13859						
		13859			13799	13799			13799	
	13269							13269		13269
		13211	13443	13385		13154	13328			
12000-12999	12539		12871	12704	12927	12485	12594	12704	12759	12649
	12110	12594	12163	12005	12058	12005	12110	12163	12059	
11000-11999		11953								11953
	11345	11345	11345	11345	11345	11345	11345	11345	11345	11345
10000-10999	10817			-	-	-	-	-	-	-
		10358	10358							
9000-9999	9915	9915	9915	9915	9915	9915	9915	9915		9915
	9095		9028							
8000-8999	-	8766	-						8766	8766
						8383	8512	8449		8090
				8144	8144					
7000-7999		7983	7983						7929	
	7417	7417		7417	7417	7417	7417	7417	7417	7417
		7055		7055				7055		
			7091							
6000-6999				6986					6986	6986
	6848	6581	6584				6917	6780	6356	
	6324	6324	6324	6324	6324	6324	6324	6324		6324
5000-5999	5633	5633	5633	5633	5633	5633	5633	5633	5633	5633
	5327	5327		5327	5327	5327	5327	5327	5327	5327
								5070	5070	
	5051									5051
		5032	5382							
			5166							
4000-4999	4842	4842	4842	4842	4842	4842	4842	4842	4842	4842
	4570	4570	4570	4570	4570	4570	4570	4570	4570	4570
	4331	4331	4331	4331	4331	4331	4331	4331	4331	4331
	4088	4088	4088	4088	4088	4088	4088	4088	4088	4088
3000-3999	3910	3910	3910	3910	3910	3910	3910	3910	3910	3910
	3741	3741	3741	3741	3741	3741	3741	3741	3741	3741
	3632	3592		3513	3526			3526	3501	3462
	3386			3386	3386	3386	3386			
	3204	3204	3204	3204	3204	3204	3204	3204	3204	3204
	3044			3044	3044	3044	3044	3044	3044	3044
2000-2999	2905	2905		2905	2905	2905	2905	2905	2905	
	2728	2728		2728						
					2746	2746	2746	2746	2746	2746
	2569	2569	2569	2569	2569	2569	2569	2569	2569	2569
				2349	2349	2349	2349	2349	2349	2349
	2257						2257	2257		
	2133	2235		2242	2249		2119	2242	2227	2220
		2119		2105	2126			2126	2098	2112
1000-999	1976				1976	1976	1976	1976		1976
	1889	1989								
	1669									



Photograph 2: Agarose gel electrophoresis of DNA fingerprinting by HindIII restriction endonuclease

antimicrobial resistance (resistant for 60% of the tested antibiotics). All tested antibiotic resistant *S.* Typhimurium isolates carried a large, 60 MDa plasmid [50]. Liebana *et al*. [51] reported that the majority of *Salmonella* serotype Typhimurium strains carry the serotype–specific plasmid of 60 MDa, alone or in combination with other plasmids. The large plasmid conferred resistance to gentamicin plus tetracycline, to gentamicin alone, or amoxicillin alone [52]. Plasmid profiling seems to present a reasonable degree of sensitivity when applied to *Salmonella* serotype Typhimurium [53]. In this study, the 11425 bp plasmid was common to all *S.* Typhimurium isolates.

The eradication of *Salmonella* isolates from the environments is practically impossible, therefore the development of preventative and control measures are necessary including improved diagnostics and hazard analysis, modern typing methods are based on characterization of the genotype of the organism. The basic premise of these typing systems is that epidemiologically related isolates are derived from the clonal expansion of a single precursor and share characteristics that differ from those of epidemiologically unrelated isolates. The usefulness of a particular characteristic (phenotypic or genotypic) for typing is related to its stability within a strain and its diversity within the species, reflecting the evolutionary genetic diversity arising from random, non lethal mutations over time. Such mutations can be detected if they are seen to occur within a restriction site that determines a DNA fingerprint [51].

The total *S.* Typhimurium DNA was extracted as described by Scheppler *et al*. [20]. DNA was digested with HindIII restriction enzyme [18] and analyzed by electrophoresis on submarine ethidium bromide containing 0.7% agarose gels. The purpose of this study was to investigate clonal diversity among poultry *Salmonella* serotype Typhimurium isolates from a variety of geographical areas at Egypt. In the present study, 24-34 bands were identified between *S.* Typhimurium isolates as shown in Table 5, photo 2. 10 bands were common to all investigated strains with molecular size 11345, 5633, 4842, 4570, 4331, 4088, 3910, 3741, 3204 and 2569. Additionally, 9 strains shared 4 common fragments at 9915, 7417, 6324 and 5327. eight and six strains had 2 common bands at 3044, 2905 and 2746, 1976, respectively; while 7 strains shared band at 2349 indicating a possible link between these strains. Mhand *et al*. [23] concluded that *S.* Typhimurium isolates were highly related or identical, differing only one band and were genetically unrelated. Malorny *et al*. [53] recorded that molecular typing has indicated that *S.* Typhimurium DT104 isolates are highly clonal. Band differences were identified between the examined strains as shown in Table 5. So, it is difficult to comment on the significance of the variety of individual sporadic clones found in the study. It seems likely that with a wider sample, these clones may be shown to be widely distributed among host and locations. Mdegela *et al*. [54] demonstrated different ribotypes using HindIII restriction endonuclease. Ten different genotypes were identified among the 140 serotype Typhimurium isolates digested with XbaI, with B1nI digestion, 11genotypes were identified. After restriction with B1nI, subgroups could be identified within the XbaI T8 genotype: T8/T8 and T8/T12, all the isolates of slaughterhouse A2 that were susceptible to all the antibiotics tested were clustered together in a subgroup of T8 with genotype T8 (XbaI) combined with T12 (B1nI), after B1nI digestion, two subgroups could be distinguished within genotypes T2 (T2/T2 and T2/T2a), T4(T4/T4 and T4/T4a) and T11(T11/T11 and T11/T11a), as recorded by Botteldoorn *et al*. [28].

In conclusion, epidemic or endemic salmonellosis in birds causes tremendous problems for the food industry and creates a potential health hazard for humans. Salmonellosis in humans can produce symptoms ranging in severity from gastric distress to death [55]. Prophylactic work based on epidemiological surveys to find the reservoirs are given high priority. Outbreak strains have traditionally been traced by several different methods, including serotyping, biotyping, phage typing and antimicrobial susceptibility testing. These methods were

recently compared with plasmid profile analysis in an investigation of well-documented *S.* Typhimurium. Plasmid profile analysis was found to be at least as good as the traditional methods and this approach has been used in the characterization of other species.Restriction enzyme analysis indicated that *S.* Typhimurium isolated from different poultry origin and geographical area are highly clonal, but it should be used in parallel with antibiotyping and plasmid analysis. Such approach would allow the establishment of compatible databases for human and veterinary isolates, which would facilitate enormously the investigation of outbreaks and the prospective use of surveillance data.

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