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Molecular epidemiology of *Salmonella enteritidis* phage type 1b and 6a isolates in Portugal

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SUMMARY

Salmonella enterica serotype Enteritidis is an important serovar comprising 76% of salmonella isolates in Portugal in 2001.

For better understand the epidemiology of salmonellosis, a total of 47 isolates of *S. Enteritidis* phage type (PT) 1b and 6a were analysed by pulsed-field gel electrophoresis (PFGE) and genomic DNA was subjected to macro restriction with *Xba*I. For PT1b isolates, only three different patterns were observed, and PT6a showed a total of 10 digestion patterns. Curiously, the main pattern among PT1b isolates seams quite similar to main pattern of PT6a isolates, but when the two patterns were analysed with Bionumerics, we observed that they exhibited some differences. It was concluded that, in 2001, there was one predominant pattern for PT1b and PT6a and, possibly, we were in presence of clonal strains that exists all over the country.

INTRODUCTION

During the last few years, salmonella has been one of the most important causes of bacterial enteric illness in humans and animals and has become an increasing public health problem in Portugal and all over the world [1, 2].

According to previous studies, *Salmonella enteritidis* accounted for more than 50% of all enteric infections caused by this species in Portugal in the last few years. This increase has made serotyping alone inadequate for epidemiological purposes and the surveillance of *S. enteritidis* depends principally, in most countries, on the use of phage typing [3–5]. The antibiotic resistance is also a very useful phenotypic discriminatory tool and is routinely performed in several reference laboratories for characterization of salmonella isolates.

Because of the increasing role of *S. enteritidis* in salmonella infections, establishment of molecular typing data is important. This may be useful in recognizing and identifying the infectious strains which cause the food-borne outbreaks or sporadic cases. Methods like phage typing, pulsed-field gel electrophoresis [6–11], plasmid profile [12] and ribotyping [6, 13] are frequently used.

Pulsed-field gel electrophoresis (PFGE) has been shown to be highly effective for epidemiological studies of some serotypes of salmonella [14–17]. The major disadvantage of this method is its labour-intensiveness and a fact that a week may be required until results are available, however some authors described a rapid PFGE protocol for Gram-negative organisms that yield results in 3 days [18].

In the present study we performed phage typing [5] and further we applied pulsed-field gel electrophoresis [11] to analyse detailed molecular relationships between strains with the same phage types, PT1b and PT6a which are the predominant phage types in Portugal.

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MATERIAL AND METHODS

Bacterial strains

A total of 673 salmonella samples (576 from humans and 97 from food) were isolated in different parts of Portugal in 2001. The human isolates were from sporadic clinical cases. The food isolates consisted of 43 samples of cooked meats, 20 egg isolates and 34 poultry isolates. All food isolates were from sporadic cases.

Isolates were identified as salmonella species using triple sugar iron agar (TSI, DIFCO, USA) or the Api ID32E Kit (Biomérieux SA, France, 32400).

Serotyping

All isolates were serotyped using the Kauffmann–White scheme [19].

Phage typing

Phage typing was done by Ward et al. method [5], and phages were obtained from the World Health Organization (WHO) International Center for Enteric Phage Typing, London, UK.

Strains that were lysed by a number of the phage but had phage lysis patterns that did not conform exactly to any of the phage lysis patterns of the known type strains were designated RDNC, for 'reacted, but did not conform' [5]. Strains that did not react with any of the typing phage were designated as 'untypeable' (UT).

Pulsed-field gel electrophoresis

Strains of *S. enteritidis* were grown on agar plates and incubated at 37 °C overnight (no longer than 24 h).

Genomic DNA was prepared and stored according to the procedures of [18].

The restriction digestion was performed by placing a slice of each plug into distilled water, restriction buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, 50 mM NaCl) and 20 units of *Xba*I (Amersham Pharmacia Biotech, E1093Y). The digestion was carried out at 37 °C for 4 h.

Samples were electrophoresed with lambda ladder PFGE marker (New England BioLabs, NO340S) as molecular size standard and with *S. braenderup* reference strain as global reference pattern (as in the Pulse-Net). PFGE was performed using a CHEF DRII system (Bio-Rad) at 6 V/cm for 22 h, and pulse times were ramped 2·2–54·2 s during the run. After electrophoresis, PFGE gels were stained with ethidium bromide and photographed with Gel Doc 2000 using the Quantity One Software (Bio-Rad, Hercules, CA, USA). Images obtained by GelDoc 2000 using Quantity One software were saved in TIFF format and transferred to the BioNumerics software (Applied Maths, Kortrijk, Belgium) for computer analyses. Similarity between fingerprints was determined by the Dice coefficient. A band position tolerance of 3% was used for analysis of PFGE patterns. Capital letters were used to designate each different pattern of *S. enteritidis* isolates in dendrogram.

Table 1. *Distribution of serotypes of* salmonella *isolates (from human and food) in Portugal in 2001*

	Human isolates		Food isolates	
Serotype	Frequency	%	Frequency	%
S. enteritidis	438	76·0	44	45·4
S. typhimurium	61	10·6	9	9·3
S. O: 4,5: i: -	27	4·7	6	6·2
S. istanbul	5	0·9	4	4·1
S. essen	5	0·9	2	2·1
S. virchow	3	0·5	1	1·0
S. derby	3	0·5	4	4·1
S. typhi	3	0·5	0	0·0
S. heidelberg	2	0·4	2	2·1
S. newport	2	0·4	2	2·1
S. brandenburg	2	0·4	0	0·0
S. agona	1	0·2	1	1·0
S. kallo	1	0·2	3	3·1
S. lagos	1	0·2	1	1·0
Others	22	3·6	18	18·5
Total	576	100·0	97	100·0

RESULTS

Serotyping

The serovar distribution of the 576 human and 97 food isolates in 2001 is shown in Table 1. Most of the clinical strains (89%) were isolated from faecal specimens of patients with diarrhoea. In 2001, 76% ($n=438$) of the isolated strains from humans were *Salmonella enterica* serovar Enteritidis. The same happened with food isolates, 45·4% ($n=44$) were *S. enteritidis*. This is also the predominant serovar in all of the different types of food isolates analysed (cooked meat, eggs and poultry).

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Table 2. *Distribution of phage type of* S. enteritidis *isolates* (*from human and food*) *in Portugal in 2001*

	Human isolates		Food isolates	
Phage type	Frequency	%	Frequency	%
PT1b	176	40·2	11	25·0
PT6a	31	7·1	4	9·1
PT1	28	6·4	8	18·2
PT4	26	5·9	0	0·0
PT20a	22	5·0	0	0·0
PT4b	21	4·8	0	0·0
PT14b	12	2·7	2	4·5
PT20	6	1·4	2	4·5
UT	30	6·8	7	16·0
Others	86	19·6	10	22·7
Total	438	100·0	44	100·0

Phage types of Enteritidis

In the past, the very low number of phage types may have depended on the low number of isolates, however when the number of isolates increased the number of phage types also rose.

The PT distribution of *S. enteritidis* isolates from humans in 2001 is shown in Table 2. PT1b was the predominant PT in both human (40·2%) and food (25%) isolates, followed by PT6a in humans (7·1%) and PT1 in food (18·2%). Of the 438 *S. enteritidis* human isolates, 30 were designated UT. In food we had 7 UT samples.

Pulsed-field gel electrophoresis

To discriminate further among isolates with the same PT (PT1b and PT6a), PFGE was applied to 47 isolates of *S. enteritidis* selected to represent predominant PT in both human and food isolates.

When chromosomal DNAs from the 47 *S. enteritidis* strains were analysed by PFGE, a total of 13 different patterns were identified (Fig. 1).

PT1b isolates showed only three different patterns (Fig. 1) although their geographic locations were distant. In this phage type human and food isolates revealed similar PFGE patterns (pattern A1b), indicating a possibly relationship between strains and perhaps a common source of infection. The food isolates were from cooked meat, eggs and poultry samples. B1b and C1b patterns belong to human isolates.

S. enteritidis PT6a showed 6 independent patterns and 4 pattern groups (Fig. 1). Four food isolates with this phage type were analysed and two of them revealed independent PFGE patterns (patterns A6a and B6a). Pattern A6a belongs to an egg isolate and B6a to a poultry isolate. The other two food isolates were from poultry and belongs to D6a pattern. This also suggests a common source of infection.

All isolates analysed were from sporadic cases, however the similarity between patterns of human and food isolates might indicate a relationship.

DISCUSSION

Salmonella infections have increased considerably in recent years in Portugal as well as in many European countries. This pathogen remains the most common cause of gastroenteritis in humans and continued surveillance is therefore necessary [20]. Phage typing has become a useful method for the demonstration of epidemiological associations between strains of *Salmonella* serovar Enteritidis and most reference laboratories now use the phage typing scheme of Ward et al., allowing the comparison of results in different countries [21].

Molecular typing techniques, such as plasmid profiles [22] or ribotyping [23], have been shown to be useful to subtype *Salmonella enteritidis*. However a high clonality between the strains has been shown and these methods do not always give enough information for epidemiological purposes [8].

The development of PFGE and publication of consensus guidelines to interpret adequately the restriction profiles [24] are useful to extend this technique for international use [8, 25].

In the present study we report the use of PFGE in salmonella isolates (human and food isolates) on the basis of phage types (PT).

Phage typing showed that *S. enteritidis* PT1b was the most prevalent among those found in human and food samples. PT 6a was the second most common phage type and curiously PT4, the predominant type in many European countries [26–28], appears only in fourth place in these isolates. Concerning food isolates, PT1 was the second phage type most significant in Portugal and, in Denmark, England and Spain it was the most predominant type among non-human strains [22, 29, 30].

The other phage types were detected in comparatively low rates and seem to play a minor role in enteric infections. PT8, which has a highlight place in isolates of United States [31] and European countries like Denmark and England, was not detected in this

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Fig. 1. Dendrogram based on PFGE profiles (with *Xba*I) of *S. Enteritidis* phage type 1b and 6a isolates from human and food.

study. Curiously it was not a common PT in Spain either [30].

Pulsed-field gel electrophoresis has been shown to be highly effective for epidemiological studies of some serovars of salmonella [14–17] and this method was used in order to epidemiologically investigate *S. enteritidis* that were derived from sporadic cases in Portugal during 2001.

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In *Salmonella enteritidis* PT1b we identified PFGE patterns quite similar on isolates from human and food origins. This suggests that some of sporadic human salmonella infections in Portugal are due to consumption of contaminated food. In this phage type we observed three different digestion patterns (A1b, B1b, C1b), though they differ only in one DNA band, and based on Tenover criteria [24] we can concluded that patterns were closely related.

Food samples belong to pattern A1b and patterns B1b and C1b are exclusive of human isolates. The three patterns are also very similar to patterns of *S. enteritidis* PT1 found in Denmark, England and Spain [21].

In those countries most of the human, food and animal isolates showed the same profiles. This fact confirms the close genetic relation of this phage type among the different links of the food chain and its spread through several countries [8, 9, 11, 16, 20]. When we analysed PT6a isolates, we observed 10 different patterns (A6a to J6a). In this phage type, and based on Tenover criteria [24], we also associated some different patterns in one main cluster (C6a, D6a, E6a, F6a, G6a and H6a) and concluded that they were closely related. We observed three more independent digestion patterns (A6a, B6a and J6a) and one other group with only two strains (I6a).

Two food isolates with this phage type exhibited independent PFGE patterns (A6a and B6a). Profiles of the other two isolates belong to cluster D6a. When we compared Portugal patterns of this phage type with patterns of Denmark, England and Spain we observed that they were quite similar and, once again, the relationships between strains of different sources and countries were confirmed.

At first sight, PT1b and PT6a, profiles seem to be quite similar, however their comparison with Bionumerics demonstrate some evident differences.

In conclusion, combination of phage typing and pulsed-field gel electrophoresis, using *Xba*I as restriction enzyme, seems to be of value in the epidemiological study of salmonella and surveillance and may be suitable for studies investigating the international distribution of clones. With regard to future work, it would be of interest to develop standardized protocols and procedures for computerised data analysis, so that international databases could be set up and used for epidemiological surveillance and control of the spread of this important pathogen.

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