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Molecular epidemiology of *Salmonella enterica* serovar Agona: characterization of a diffuse outbreak caused by aniseed-fennel-caraway infusion

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SUMMARY

During 2002–2003 increased numbers of notified salmonellosis due to *S. enterica* serovar Agona were observed in Germany. In order to understand the recent spread of this serovar and to trace the route of infection to its source, a new phage-typing scheme and pulsed field gel electrophoresis (PFGE) were used to analyse these isolates. By using 14 bacteriophages, 52 phage types were distinguished among the *S.* Agona strains. PFGE also differentiated 52 different patterns. A combination of both methods generated 94 clonal types among 165 *S.* Agona strains originating from Germany and other countries including the United States, United Arab Emirates, Turkey, India, Austria and Finland, indicating a great biological diversity within this serovar. However, 36 recent *S.* Agona isolates from infantile gastroenteritis in Germany, from an untreated batch of aniseed imported from Turkey and from fennel-aniseed-caraway infusion (packed in tea bags) revealed clonal identity indicating their epidemiological relatedness as a new source of infection. It is suggested that strains of *S.* Agona will continue to be of public health concern, and that phage typing together with PFGE typing should be applied as reliable and rapid tools for epidemiological subtyping and future monitoring.

INTRODUCTION

Human salmonellosis is still one of the most frequently occurring foodborne diseases worldwide. Food of animal origin, such as eggs and chicken, pork and beef and their respective products have been identified as the primary vehicles for *Salmonella*, in particular strains of *S. enterica* serovar Enteritidis and Typhimurium [1].

However, due to changing food production, consumption habits and the global food trade, new vehicles for *Salmonella* infection have been recognized, such as sprouts, spices (paprika powder), and chocolate [2–9], and *Salmonella* serovars other than *S.* Typhimurium and *S.* Enteritidis have been identified as aetiological agents, including *S.* Saintpaul [5],

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S. Bovismorbificans [5, 6], *S.* Oranienburg [8, 9], *S.* Rubislaw [5] and *S.* Agona [2].

Recently, 42 cases of infantile salmonellosis associated with serovar *S.* Agona were observed in Germany among infants aged <13 months [10] (J. Koch et al., unpublished observations). *S.* Agona is not frequently identified as a cause of human salmonellosis in Germany although in the 1970s this serovar was of public health concern in Germany, United States, United Kingdom and The Netherlands [11] due to contaminated Peruvian fishmeal infecting domestic animals, followed by increasing numbers of infections among humans [12].

In order to understand the recent increased incidence of *S.* Agona infections in Germany and to identify the source of the infections, we have applied pulsed field gel electrophoresis (PFGE) and a newly developed phage-typing scheme. These methods were validated using previously isolated strains from sporadic case between 1997 and 2003, outbreak strains from the 1970s, strains from other countries, and strains from sources other than human gastroenteritis (fennel, aniseed, pork meat, egg, soil, cattle, dogs, falcons, etc.). A clonal identity between *S.* Agona isolates from infantile gastroenteritis and aniseed-fennel-caraway infusions (packed in tea bags) points to a common source of infection (J. Koch et al., unpublished observations).

MATERIALS AND METHODS

Bacterial strains

A total of 165 strains of *S.* Agona were included in this study (Table 1). They were submitted during the period 1969–2003 to the National Reference Centres for *Salmonella* in Germany, and originated from outbreaks and sporadic cases of gastroenteritis in humans (96), animals (e.g. cattle, pig, chicken, turkey, camel, bird and dog) (26), food (23), feed (14) and the environment (6). Additionally, a *S.* Agona reference strain (PHLS, England) was used for comparative purposes. All strains were stored as glycerol (20%) cultures at -70 °C.

Isolation of typing phages

Fourteen typing phages were isolated from *S.* Agona strains. Phages 1 and 4 were obtained by direct isolation from two of 80 lysogenic strains, while the other 12 represented adaptations of phages from four *S.* Agona isolates of various origin. The adaptation and propagation of bacteriophages were carried out according to Adams [13] and Ward et al. [14]. The isolation of the respective bacteriophages was performed by mitomycin induction [15]. Strains were grown at 37 °C for 5 h with agitation in 10 ml double-strength Bacto nutrient broth (Becton Dickinson GmbH, Heidelberg, Germany) containing 2 μg mitomycin/ml. After centrifugation at 8000 rpm for 10 min, the supernatants were carefully removed and sterilized by passage through a Millipore filter (0·6 μm pore size).

The adaptation of phage [14] to various *S.* Agona strains was carried out by spotting several dilutions of the phage on different strains plated on nutrient agar. From each culture a single plaque was removed and inoculated into 2 ml broth, followed by incubation for 5 h at 37 °C. The propagated phages, which represent new lines of phages adapted by restriction/modification or phage module exchange, were removed in the supernatant after centrifugation and titrated on the respective sensitive strains.

Phage typing

Routine test dilutions of each of the typing phages were applied to Nutrient Difco agar plates with a lawn of the respective bacterial reference strains using a multipoint inoculator. They were incubated overnight at 37 °C until phage lysis could be observed. The phage patterns (phage types) and readings are summarized in Table 2. Some of the isolates gave rise to lysis reactions (sometimes weak) with the typing phages (see Table 2).

PFGE

Genomic DNA embedded in agarose was prepared as previously described [16] with minor modifications. PFGE was performed on CHEF DRIII (Bio-Rad, Munich, Germany) at 14 °C in 1·2% agarose gels for 40 h with a constant voltage of 200 V. For DNA restricted with *Xba*I and *Bln*I pulse times ranged from 2 to 20 s during the first 15 h, from 20 to 50 s for the following 15 h and from 50 to 80 s for the final 10 h. The pulse times for *Spe*I were from 2 to 10 s during the first 15 h, from 10 to 30 s for the following 15 h and from 30 to 60 s for the final 10 h. The reading and interpretation of the PFGE pattern was carried out according to Claus et al. [17] using the RFLPScan™ System (Scananalytics, Richmond, USA). Chromosomal DNA of *S.* Braenderup (Salm-Gene project, reference strain H9812) digested with *Xba*I was used as a molecular size

Table 1. *Year of isolation, number and epidemiological origin of* S. *Agona strains investigated in this study*

Year of isolation	Number of *S.* Agona strains tested	Origin
1969	1	Human, Germany
1975	1	Water, Germany
	2	Human, Germany
1994	3	Fishmeal, Norway, Peru, Chile
	1	Food, Germany
1996	3	Single cases of gastroenteritis, Germany
	2	Food, Germany
	1	Feed, Germany
	1	Cattle, Germany
	3	Poultry, Germany
	1	Feed, Germany
1997	1	Single cases of gastroenteritis, Germany
	8	Single cases of gastroenteritis, Germany
	1	London reference strains
	1	Spice, Turkey
1998	1	Chicken, Germany
	4	Pig, Germany
	1	Meat, Germany
	2	Turkey, Germany
1999	7	Single cases of gastroenteritis, Germany
	1	Animal, Dubai
	1	Hygenic swab, Germany
	3	Human outbreak, Vaara, Finland
	1	Feed, Germany
2000	7	Single cases of gastroenteritis, Germany
	1	Cattle, USA
	2	Chicken, USA
	1	Pig, USA
	1	Human, Finland
	1	Camel, Dubai
	1	Animal, India
	1	Dog, Germany
2001	4	Single cases of gastroenteritis, Germany
	2	Spices curry, peppermint leaves, Germany
	1	Feed, Germany
	1	Bird, Dubai
	9	Outbreak strains, Germany
	10	Single cases of gastroenteritis, Germany
	1	Aniseed imported, Germany
	3	Feed, Germany
2002	1	Surface water, Germany
	2	Meat, Germany
	1	Pig, Germany
	1	Poultry, imported
	2	Egg white powder, Germany
	1	Garlic powder, Germany
2003	36	Single cases of gastroenteritis, Germany
	2	Single cases of gastroenteritis, Austria
	6	Fennel aniseed infusions of different companies, Germany
	2	Aniseed, imported from Turkey, manufactured in Germany
	2	Single cases of gastroenteritis, Austria
	2	Calf, Germany; cattle, Austria
	4	Coarse colza mail, different companies, Germany
	1	Liquid manure, Austria
	1	Falcon, Dubai
	1	Composted soil, Germany
	1	Pork, Germany
	1	Turkey meat, Germany
	1	Chicken, Austria

marker [18]. The PFGE patterns were designated arbitrarily by numbering if they differed by more than four fragments; letters (a, b, c) in addition to the numbers were used to designate closely related patterns differing only by 1 or 2 fragments. For international comparison the PFGE patterns have been finally designated as SAX001, SAX002, etc. for *S.* Agona digested by *Xba*I, SAB001, etc. for *S.* Agona digested by *Bln*I, and SAS001, etc. for *S.* Agona digested by *Spe*I.

Designation of clonal types

Clonal types were defined arbitrarily by numbers on the basis of PFGE patterns (SAX001, SAX002, etc.) and phage types (PT1, PT2, etc.) as described earlier [5, 16]. For example, clonal type 1-2 indicates a distinct PFGE pattern designated arbitrarily as SAX001 and a distinct phage type designated PT2 (see Table 3).

RESULTS

Phage types

A broad spectrum of 52 phage types (PT) for *S.* Agona was established, indicating the usefulness of the

Table 2. *Phage-typing scheme of* S. *Agona*

Strain no.	PT	1	2	3	4	5	6	7	8	9	10	11	12	13	14
15228/97	1	OL	OL	OL	OL	OL	SCL	OL	OL	OL	OL	OL	OL	OL	−
4005/03	2	OL	OL	OL	++	SCL	+++	OL	OL	OL	OL	+	OL	−	+
1043/75	3	OL	OL	OL	+++	+++	+++	OL	OL	OL	OL	SCL	OL	−	+++
1223/98	4	OL	OL	OL	++	SCL	SCL	SCL	SCL	++	+	++	+++	−	−
8777/99	5	OL	OL	OL	OL	+++	SCL	+	OL	OL	OL	SCL	OL	−	++
605/03	6	OL	OL	OL	OL	+++	+++	OL	++	++	++	+++	−	−	+
198/99	7	++	OL	OL	+++	+++	++	SCL	++	OL	OL	+	OL	−	+
2/69	8	OL	OL	OL	SCL	+++	+++	SCL	OL	OL	OL	−	OL	OL	++
761/97	9	OL	OL	OL	SCL	++	++	SCL	OL	OL	+++	−	−	−	++
2622/94	10	OL	OL	++	+++	+++	+++	SCL	SCL	++	−	−	−	−	+
1676/00	11	OL	++	OL	OL	+++	SCL	OL	OL	−	−	++	−	OL	++
10892/02	12	OL	OL	++	++	+++	+++	OL	OL	−	−	−	−	−	−
277/98	13	OL	OL	+	SCL	++	++	SCL	−	+++	−	+++	−	−	+
6755/00	14	OL	OL	OL	++	+++	+++	OL	−	++	++	+++	−	−	−
4296/96	15	OL	OL	OL	OL	+++	+++	OL	−	−	−	−	−	−	+
7898/98	16	OL	OL	OL	OL	+++	+++	−	OL	OL	−	+	−	+++	+
758/97	17	OL	OL	OL	OL	+++	SCL	−	++	−	+	SCL	++	−	+
10944/00	18	OL	OL	OL	+++	+++	++	−	−	−	−	−	−	−	++
3665/02	19	OL	OL	OL	++	++	OL	−	−	−	−	−	−	−	++
3517/03	20	OL	OL	OL	+++	SCL	−	OL	OL	OL	OL	−	OL	−	−
8765/98	21	OL	OL	OL	OL	−	+++	OL	OL	OL	OL	−	OL	OL	++
3564/03	22	OL	OL	OL	+++	++	−	++	OL	++	−	−	−	OL	+
122/03	23	OL	OL	OL	+++	++	−	SCL	−	−	−	−	−	−	−
7203/99	24	OL	OL	OL	OL	+++	−	−	++	−	−	−	−	−	+++
439/03	25	OL	OL	OL	+++	+++	−	−	−	−	−	−	−	−	−
4682/03	26	OL	OL	OL	OL	+	−	OL	OL	+++	OL	−	OL	−	+
405/02	27	OL	OL	OL	SCL	−	SCL	−	−	OL	−	SCL	−	−	−
2980/00	28	+++	OL	OL	OL	−	−	++	+	+	++	−	++	−	−
7266/98	29	+++	+++	OL	+++	−	−	−	−	−	−	−	−	−	−
3570/03	30	OL	+++	OL	−	+++	−	OL	OL	OL	OL	−	OL	−	−
10091/02	31	OL	OL	++	−	++	+++	OL	+	−	+++	+++	−	−	++
6684/03	32	OL	OL	OL	−	++	++	OL	OL	OL	OL	+	OL	−	−
2780/00	33	OL	OL	OL	−	+++	−	−	−	−	−	−	+	−	−
244/03	34	OL	OL	OL	−	−	−	−	−	−	−	−	−	−	−
6498/98	35	OL	SCL	++	+	−	−	−	−	−	−	−	−	−	−
4490/99	36	OL	OL	−	OL	+++	+++	++	−	−	−	−	−	−	+
3579/03	37	OL	OL	−	−	++	−	−	−	−	−	−	−	−	+
3575/03	38	SCL	+	OL	−	+++	++	−	−	+	−	−	−	−	−
637/03	39	OL	−	OL	−	SCL	SCL	−	++	OL	−	SCL	+	OL	−
2189/02	40	OL	−	OL	−	−	−	−	−	+	+	−	+	−	−
10949/00	41	OL	−	OL	−	−	−	−	−	−	−	−	−	−	−
7694/99	42	OL	−	+	+++	++	−	−	−	−	−	−	−	−	−
31/75	43	OL	−	−	+++	+++	+++	−	+++	−	−	OL	−	−	++
4263/02	44	++	−	−	−	−	−	−	−	−	+++	+++	−	−	−
10947/00	45	−	OL	OL	OL	−	−	−	−	+	−	−	−	−	−
1887/02	46	−	SCL	−	+++	−	SCL	−	−	−	−	SCL	−	−	+
1275/00	47	−	OL	−	OL	−	−	−	+++	−	−	−	−	−	−
6497/98	48	−	OL	−	+++	+	−	−	−	−	−	−	−	−	−
2793/96	49	−	−	−	+++	−	−	−	−	−	−	−	−	−	−
3695/98	50	−	−	−	+++	−	−	−	++	−	−	−	−	−	−
3863/03	51	−	−	−	−	+++	−	−	−	−	−	−	−	−	−
6719/94	52	SCL	−	−	−	−	−	−	−	−	−	−	−	−	−

OL, Opaque lysis; SCL, semi-confluent lysis.
+++, 80–100 plaques; ++, 40–80 plaques; +, 20–40 plaques; −, no reaction.

Table 3. *Clonal types and properties of* S. *Agona isolates*

Clonal type	Year of isolation	Number of isolates tested	Source
1-2	2002/2003	41	Outbreak, Germany
1-2	2002/2003	9	Aniseed-fennel infusions (6), aniseed (3)
1-2	2002/2003	2	Surface water, Germany; human, Austria
2-2	2003	1	Outbreak Hildesheim, Germany
9-19	2001/2002	3	Spice, curry (1), meat (2), Germany
11-34	2003/2001	4	Human (3; 2003), Germany
12-34	1994	1	Fish meal, Peru
12-52	1994	2	Fish meal, Norway, Chile
13-27	2002	1	Garlic powder, Germany
25-8	1969	1	Human, Germany
34-47	1998/2000	3	Pig (1), human, Germany
38-16	1998	1	Spice, Ankara, Turkey
47-20	2001	1	Peppermint leaves, Germany
Others*	1975, 1994; 1996–2003	95	Human, animals, food, feed, environment

* Others: 1-5, 1-6, 1-12, 3-5, 3-37, 4-20, 5-5, 5-19, 5-23, 5-30, 5-44, 6-40, 7-5, 7-23, 7-37, 7-40, 8-7, 9-32, 10-39, 11-26, 11-29, 12-rough, 12-36, 12-44, 14-32, 14-5, 14-7, 14-31, 15-49, 15-47, 16-5, 16-15, 17-5, 17-8, 17-34, 17-46, 18-37, 19-22, 20-51, 21-38, 22-ut, 23-34, 23-35, 24-46, 25-4, 25-10, 25-12, 26-15, 27-5, 27-8, 28-26, 29-41, 30-9, 30-11, 31-45, 32-28, 32-41, 33-5, 33-25, 34-35, 34-50, 35-18, 35-2, 35-3, 36-48, 37-5, 37-13, 39-21, 40-5, 40-43, 41-42, 42-5, 43-5, 44-47, 45-2, 46-2, 48-25, 49-1, 49-20, 50-24, 51-5, 51-47, 52-14.

Table 4. *Comparison of phage types identified among PFGE XbaI types of* S. *Agona*

Phage type (PT)	PFGE types (see Fig. 2)
PT2	1, 1a, 1b, 1d, 1e, 2, 35, 45, 46
PT5	1d, 3a, 5, 7, 14, 16, 17a, 27, 33, 37, 40, 42, 43, 51
PT20	1d, 4, 47, 49
PT23	5, 7
PT25	33a, 48
PT34	11a, 12, 17, 23
PT37	3, 7, 18
PT47	15, 34a

system for epidemiological work (Table 2). All phage-type reference strains were defined by PFGE (Table 3). Only one strain out of the 165 *S*. Agona strains under investigation was found to be untypable (ut) by phage typing (clonal type 22-ut, Table 3), although it remained sensitive to the *Salmonella* phage O1 (ut/O1+). One strain became rough (clonal type 12-rough, Table 3) and reacted with the rough phage London (R_{LO}). Some phage types of *S*. Agona could be further distinguished by application of PFGE (Table 4).

PFGE types

Fifty-two different PFGE patterns were identified following *Xba*I digestion among the *S*. Agona strains (Fig. 1, Table 3). A total of 112 out of 165 represented each separate PFGE type (Table 3) indicating high diversity. The most frequently occurring PFGE type, designated SAX001, was identified among 41 of the 165 strains tested (Table 3). SAX001 appeared with variations of one or two bands which were designated SAX001a, b, c, d, and e respectively (see Fig. 2). In order to confirm that these pattern variations following *Xba*I digestion were indeed related to SAX001, the respective strains were also subjected to PFGE after *Bln*I and *Spe*I digestion (Fig. 2). Since the SAB001 and SAS001 PFGE types also contained band variations, we concluded that the SAX001 variations are highly related (Fig. 2).

Clonal analysis

Phage types and PFGE pattern were rather polymorphic properties among the *S*. Agona strains tested (Tables 2 and 3) and so both methods can be used to define clonal types for epidemiological purposes. As seen in Table 3, 94 different clones have been defined, indicating great biological diversity within this serovar. However, almost all the *S*. Agona isolates that

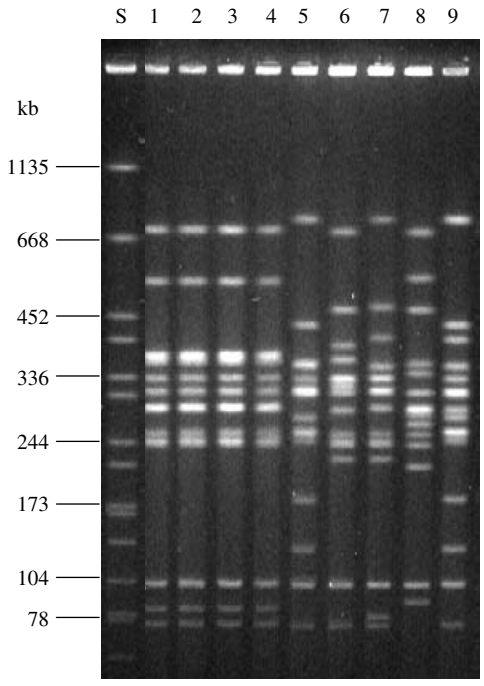
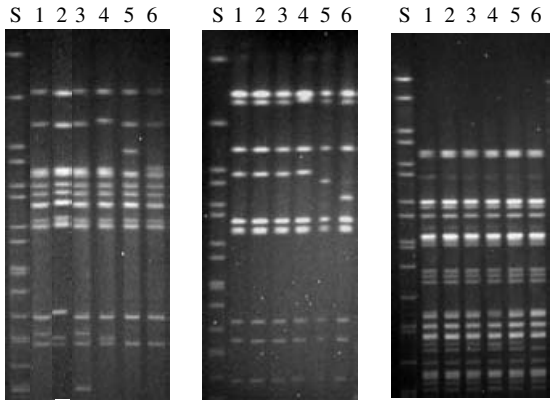


Fig. 1. PFGE pattern (*Xba*I) identified among selected number of *S*. Agona isolates. Lanes 1–4, SAX0001 (outbreak); lane 5, SAX0011a; lane 6, SAX0017; lane 7, SAX0018; lane 8, SAX0007; lane 9, SAX0015.



SAX

lane 1	1
lane 2	1a
lane 3	1b
lane 4	1c
lane 5	1d
lane 6	1e

SAB

lane 1	1
lane 2	1
lane 3	1
lane 4	1b
lane 5	1a
lane 6	1c

SAS

lane 1	1
lane 2	1
lane 3	1
lane 4	1a
lane 5	1
lane 6	1b

Fig. 2. PFGE pattern of *S*. Agona SAX0001 and variants 1a, b, c, d, e in comparison to *Bln*I(SAB)–PFGE and *Spe*I(SAS)–PFGE pattern.

originated from babies in Germany during 2002–2003, from fennel-aniseed-caraway infusions as well as from an aniseed lot belonged to the same clonal type 1-2, indicating their epidemiological relatedness.

DISCUSSION

A considerable biological diversity was detected among the 165 *S*. Agona (O:4,12 H:f,g,s:–) strains obtained from Austria, Finland, Turkey, United Arab Emirates, India, United States, and Germany. Using a new phage-typing scheme together with PFGE typing we identified 94 clonal groups (Table 3) of which 41 isolates (31·5%) belonged to the clonal type designated 1-2 (PFGE type 1, PT2). This clonal type was predominantly observed among the strains originating from an outbreak due to aniseed-fennel-caraway infusion in Germany during 2002–2003 with additional single isolates (variants PFGE type 1d, PT2) from Austria (2002, human) and from a surface water sample (2002, Germany) (Table 3). Since phage types and PFGE types among the outbreak strains remained stable even after 24 months of storage (see Material and methods section), the high clonal variability cannot be explained by genetic instability constantly giving rise to new PFGE or phage types in Europe. Therefore, all PFGE and phage-type variations can be regarded as an indication of great phylogenetic diversity of *S*. Agona in nature. However, one exception was observed in one isolate of an outbreak in Hildesheim, Lower Saxony (six patients), which belonged to PT2 but showed a different PFGE pattern, SAX002 (Table 3), probably due to an incoming plasmid (data not shown). None of the isolates except this SAX002 strain had antibiotic-resistance genes and plasmids; SAX002 exhibited kanamycin, streptomycin, sulphamerazin, tetracycline and trimethoprim resistance (KSSuTTp) due to a 90-kb plasmid. The generation of a new PFGE type might be due to chromosomal rearrangement after plasmid uptake. We propose the combined use of PFGE and phage typing for epidemiological surveillance and for detecting sources and routes of *S*. Agona infection (see also [5]). Furthermore, both methods enabled us also to detect 'normal' genetic variations by acquisition of plasmids and bacteriophages.

It is interesting to note that we could not trace the 1-2 clonal type to other countries or from other years of origins. However, the presence of *S*. Agona clonal type 1-2 in samples of aniseed-fennel-caraway

infusion produced by different companies and additionally in an aniseed lot imported from Turkey provides strong evidence in this case-control study that this product was the source of the outbreak (J. Koch et al., unpublished observations). Since the lot of aniseed imported from Turkey contained *S*. Agona with the same clonal identity as the outbreak isolates, it was anticipated that this clonal type would also be found among other isolates from Turkey, but this was not the case (see the differences to the single isolate from Turkey belonging to clonal type 38-16; Table 3). Nevertheless, *S*. Agona of the 2002–2003 outbreak originated from a non-animal, dry product (fennel-aniseed-caraway infusion) and is consistent with other reports of *Salmonella* in dry products [7, 19–21]. In recent years *S*. Agona has frequently been associated with dry foods, such as machacado, an air-dried raw beef product in Mexican food [22], a kosher savoury snack [23, 24], and canola and soy meal in different states of Australia [25]. Furthermore, in the United States a multi-state outbreak of *S*. Agona with 209 cases (47 of whom were hospitalized) was linked to toasted oats cereal in 1998 [26]. Recently, *S*. Agona was found in peppermint leaves, curry and garlic powder, but belonged to different clonal types (Tables 1 and 3) [22, 27]. *S*. Agona strains were also isolated from raw vegetables in Malaysia [2].

The *S*. Agona outbreak in Germany caused by a contaminated aniseed lot from Turkey, which was distributed to several aniseed-fennel-caraway infusion producers shows certain parallels to the *S*. Agona epidemic in Europe during the 1970s that was associated with contaminated imported Peruvian fishmeal [11]. The fishmeal was distributed to several European countries and subsequently infected domestic animals (pigs) before showing increased incidence in humans [12]. However, these strains were unrelated to the present outbreak (25-8; Table 3). Similarly, strains from an outbreak in Vaara, Finland [28] of unknown origin belonged to different phage and PFGE types (PFGE type SAX031 and PT45, Table 3).

In conclusion, in a world with global international food and feed trades, it seems that food surveillance should be extended to dried-food products. For *S*. Agona we suggest the use of PFGE together with the new phage-typing scheme presented here for the clonal discrimination of *S*. Agona strains (Table 3, Fig. 1) and as an efficient approach to analysing an outbreak caused by aniseed-fennel-caraway infusion in infants in Germany.

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