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Korrespondenzadresse:
stephanr@fsafety.uzh.ch

Institute for Food Safety and Hygiene, National Centre for Enteropathogenic Bacteria and Listeria, University of Zurich, Winterthurerstr. 272, 8057 Zurich, Switzerland

Characteristics of *Salmonella enterica* ssp. *enterica* serotype Schwarzengrund associated with human infections in Switzerland: 2006–2010

Charakterisierung von Salmonella enterica ssp. enterica serotype Schwarzengrund Stämmen isoliert von Patienten zwischen 2006 und 2010 in der Schweiz

Magdalena Nüesch-Inderbinen, Nicole Cernela, Herbert Hächler, Roger Stephan

Summary

Salmonellosis is a gastrointestinal infection caused by *Salmonella enterica* and commonly acquired from contaminated food. Worldwide, *Salmonella* causes millions of infections and thousands of deaths annually, posing a significant threat to public health. While *S.* serovars Typhimurium and Enteritidis are the predominant causes of nontyphoidal salmonellosis, *Salmonella* serovar Schwarzengrund has hitherto been an uncommon cause of human salmonellosis. However, recent reports suggest that this serovar is becoming more prevalent in Asia, Europe and the U.S.

A total of 21 strains isolated from different patients from 2006 through 2010 in Switzerland were characterized by (i) assessing phenotypic antibiotic resistance profiles using the disk diffusion method and (ii) by genotyping using pulsed-field gel electrophoresis (PFGE) after macrorestriction with XbaI in order to evaluate strain relationship.

The annual incidences from 2006 to 2010 of *S.* Schwarzengrund varied between 0.11/100'000 in 2006 (highest incidence, 8 cases) and 0.01/100'000 in 2010 (lowest incidence, 1case). All of the isolates were resistant to the sulfonamide antibiotic sulfamethoxazole. Two strains were resistant to the quinolone antimicrobial nalidixic acid as well as to the ß-lactam antibiotic ampicillin and were classified multidrug-resistant. Analysis of PFGE data showed high similarity between the strains, as well as similarity to common international clones of *S.* Schwarzengrund.

These findings highlight the need for continued surveillance of occurring genotypes and of antimicrobial resistance in *Salmonella* spp.

Keywords: *Salmonella* Schwarzengrund, human infections, resistance profiles, genotypes

Zusammenfassung

Die Salmonellose ist eine Magen-Darm-Infektion, die durch *Salmonella enterica* verursacht und häufig durch kontaminierte Lebensmittel erworben wird. Weltweit verursachen Salmonellen Millionen von humanen Infektionen und Tausende von Todesfällen pro Jahr. Während die Serovare *S.* Typhimurium und Enteritidis die vorherrschenden Ursachen für nontyphoidale humane Salmonellosen sind, ist *Salmonella* Schwarzengrund bisher selten. Allerdings deuten die jüngsten Zahlen darauf hin, dass in Asien, Europa und den U.S.A. die Häufigkeit dieses Serovars ansteigt.

Im Rahmen dieser Arbeit wurden insgesamt 21 Stämme isoliert zwischen 2006 und 2010 von verschiedenen Patienten in der Schweiz und durch (i) Erstellung eines Resistenzprofils mittels Agardiffusionsmethode und (ii) durch Genotypisierung mittels Pulsfeld-Gelelektrophorese (PFGE) nach einer Makrorestriktion mit XbaI weitergehend charakterisiert.

Die Inzidenz von *S.* Schwarzengrund schwankte zwischen 0.11/100'000 im Jahre 2006 (höchste Inzidenz, 8 Fälle) und 0.01/100'000 im Jahr 2010 (tiefste Inzidenz, 1 Fall). Sämtliche Isolate waren resistent gegen Sulfamethoxazol. Zwei Stämme waren resistent gegen Nalidixinsäure und Ampicillin und wurden als multiresistent eingestuft. Die PFGE Daten ergaben eine hohe Ähnlichkeit der Stämme untereinander sowie Ähnlichkeiten mit international vorkommenden Klonen von *S.* Schwarzengrund.

Diese Ergebnisse unterstreichen einmal mehr die Notwendigkeit einer Überwachung der vorkommenden Genotypen und der Antibiotikaresistenz von *Salmonellen* spp.

Schlüsselwörter: *Salmonella* Schwarzengrund, humane Infektionen, Resistenzprofil, Genotypen

Introduction

Salmonellosis is generally acquired by the consumption of contaminated food and constitutes a global burden to public health (Rabsch et al., 2001; Olsen et al., 2001), causing estimated 93.8 million human infections and 155'000 deaths annually worldwide (Majowicz et al. 2010). More than 2500 serovars of *Salmonella enterica* subsp. *enterica* have been identified. In most European countries, there are five serovars that account for the majority of cases of Salmonella in humans. These serovars are *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Hadar and *S.* Virchow. These main serovars are thought to be caused by national animal food production (Hendriksen et al., 2011). However, their distribution may vary over time and across countries (Huehn et al., 2010) and is also associated with the increased trade of food, increased travel and migration (Hendriksen et al., 2011; Schmid et al., 2013). Drugs of choice for treating severe *Salmonella* infections include fluoroquinolones, third-generation cephalosporins or trimethoprim-sulfamethoxazole (Hohmann et al., 2001). The emergence and dissemination of antibiotic resistant bacteria, including *Salmonella* (Parry, 2003), and subsequent failure of treatment of infections is a growing global public health problem.

In previous years, cases of *S.* Schwarzengrund infections have been reported increasingly in some European countries, e. g., England and Wales in 2006 (http://www.hpa.org.uk/hpr/archives/2007/hpr0407.pdf), and Denmark (Aarestrup et al., 2007). *S.* Schwarzengrund is one of the few *Salmonella* serovars known to cause invasive salmonellosis (Vugia et al., 2004) and persistent nosocomial infections (Olsen et al., 2001).

Multidrug-resistant (MDR) *S.* Schwarzengrund strains isolated from humans have been shown to spread internationally from contaminated food products (predominantly chicken) imported from Asia to Europe and the United States (Aarestrup et al., 2007). In Thailand, a world leading poultry exporter, *S.* Schwarzengrund ranks among the 20 most frequently serotyped human *Salmonella* isolates (Hendriksen et al., 2011).

From 2006 to 2010, 8175 cases of *Salmonella* infection were reported to the Swiss Federal Office of Public Health (http://www.bag.admin.ch/k_m_meldesystem/00733/00813/) and isolates were submitted to the National Centre for Enteropathogenic Bacteria for final serological identification by slide agglutination with commercial antisera according to the Kauffmann-White-LeMinor scheme (Grimont and Weill, 2007).

In this study, 21 *S.* Schwarzengrund strains that were isolated from different patients between 2006 and 2010 in Switzerland were characterized by assessing their phenotypic antibiotic resistance profiles using the disk diffusion method, and by evaluating their genetic relationships using pulsed-field gel electrophoresis (PFGE).

Methods

Human salmonellosis is a reportable disease in Switzerland. During the time span from 2006-2010, 21 human isolates of *S.* Schwarzengrund were identified and were analysed in this study. Multiple isolates from the same patient were excluded. All samples had been collected and submitted by diagnostic laboratories (n = 18) or hospitals (n = 3) throughout Switzerland.

Antimicrobial susceptibility testing

The antimicrobial resistance profiles of the isolates were investigated by the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2008) The panel of antibiotic disks (Becton, Dickinson and Company, Maryland, USA) comprised ampicillin (AM), amoxicillin/clavulanic acid (AMC), cephalothin (CF), cefotaxime (CTX), ciprofloxacin (CIP), gentamicin (GM), tetracycline (TE), streptomycin (S), chloramphenicol (C), kanamycin (K), nalidixic acid (NA), sulfamethoxazole (SMZ), and trimethoprim (TMP). The strains were classified according to the CLSI criteria (CLSI, 2008) as either resistant, intermediate or susceptible to each antibiotic agent.

Genotyping

Pulsed-field gel electrophoresis (PFGE) was performed by following the CDC PulseNet protocol (http://www.cdc.gov/pulsenet/protocols.htm) with minor modifications. In brief, strains were grown on blood agar at 37 °C over night. Colonies from blood agar were resuspended in cell suspension buffer (OD600 = 1). The bacterial cell suspension was mixed with 400 µl of 1.4 % Pulsed Field Certified Agarose (BIO-RAD, Munich, Germany) and cells were lysed by proteinase K treatment over night. After lysis the plugs were washed twice for 30 min in ultrapure water and 4 times for an hour in Tris-EDTA (TE) buffer. After washing with TE buffer, DNA agarose plugs were incubated over night in the presence of XbaI (Roche, Mannheim, Germany) following the manufacturer's instructions. Restricted DNA in plug slices was separated in a 1 % SeaKem Gold agarose gel (BioConcept, Allschwil, Switzerland) at 6 V/cm in 0.5 M Tris-Borate-EDTA buffer (12 °C) using a CHEF-DR III system (BIO-RAD, Munich, Germany). The pulse times ranged from 2 to 64 sec for 20 h at an angle of 120°. Gels were stained with ethidium bromide and visualized under UV light with Gel Doc (BIO-RAD, Munich, Germany) and analyzed with BioNumerics software Version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). Pair wise similarities between the XbaI pulsed-field patterns were calculated by the DICE's similarity coefficient. Clustering was based on the unweighted pair-group method with averages (UPGMA), setting tolerance and optimization each at 1.5 %. Isolates with PFGE patterns differing by three or less bands were designated as related (Dice coefficient of similarity of >80 %). *Salmonella* Braenderup strain H9812 (ATCC BAA 664) was selected as a reference strain.

Results

Clinical and epidemiological data

Of the 21 *S.* Schwarzengrund strains, 18 were known to originate from stool samples. Of the remaining 3 strains the origin remained unknown (Tab. 1).

In 2006, *S.* Schwarzengrund accounted for 0.5 % of the annual *Salmonella* isolates in Switzerland. The annual rates for the ensuing years were the following: 0.1 % in 2007, 0.3 % in 2008, 0.3 % in 2009, and 0.1 % in 2010. The annual incidence of *S.* Schwarzengrund in Switzerland was highest in 2006 with a value of 0.1/100'000. In 2007 the annual incidence was 0.03/100'000, followed by the incidence of 0.08/1000'000 in 2008, 0.05/100'000 in 2009, and the lowest incidence of 0.01/100'000 in 2010.

For 19 of the 21 patients, the age was known. In the age group consisting of children of 5 years or younger, 4 strains

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TABLE 1: *Resistance profiles and anamnestic data of the 21 S. Schwarzengrund isolates.*

Year of isolation	Isolate	Disk diffusion [mm]													gender	age	source	stay abroad/conspicuous feature
		AM	AMC	CF	CTX	GM	K	S	TE	CIP	NA	SMZ	TMP	C30				
2006	N06-226	25	25	25	32	17	18	15	18	32	24	6	26	26	f	27	feces	
2006	N06-569	24	26	24	30	18	20	16	19	34	25	6	32	25	m	24	feces	
2006	N06-778	24	26	23	31	20	21	16	20	36	15	6	30	29	nd	46	feces	
2006	N06-1170	26	28	26	36	20	22	16	18	36	24	6	28	27	f	24	feces	Tunisia
2006	N06-1204	6	20	22	30	12	6	12	19	28	6	6	25	14	m	7	feces	
2006	N06-1263	25	28	28	32	21	23	17	20	36	26	6	32	23	f	nd	feces	carrier
2006	N06-1324	22	28	22	26	20	22	6	22	42	28	6	28	31	m	2	feces	
2006	N06-1430	26	27	26	32	22	23	16	22	34	27	6	30	28	f	19	feces	Tunisia
2007	N07-235	24	24	25	30	19	21	16	6	25	14	6	28	24	m	65	feces	
2007	N07-411	25	26	22	26	17	17	14	20	32	22	6	26	24	f	13	nd	
2007	N08-631	5	23	23	32	17	17	14	19	27	6	6	27	26	m	37	feces	
2008	N08-1804	23	23	25	29	19	19	14	19	34	23	6	30	24	f	9	feces	
2008	N08-1849	25	24	26	28	20	19	15	17	34	23	6	28	27	f	18	feces	
2008	N08-1869	25	22	25	31	21	23	16	20	32	24	6	28	27	m	21	feces	
2008	N08-1944	24	25	26	32	19	20	16	19	34	25	8	29	27	f	35	feces	
2008	N08-1989	23	28	23	34	19	19	15	19	36	24	8	28	26	m	42	feces	Bulgaria
2009	N09-1993	24	26	23	28	19	20	14	28	38	24	10	26	24	f	1	nd	
2009	N09-2242	26	26	24	30	18	18	15	18	32	24	10	30	25	f	nd	feces	
2009	N09-2375	24	24	23	28	19	19	16	19	32	22	6	28	26	f	18	feces	
2009	N09-2585	25	25	22	29	19	19	14	19	32	23	8	27	27	m	1	nd	
2010	N10-0207	26	24	30	40	21	21	14	19	36	24	6	25	25	m	3	feces	Brasil

Isolates resistant, intermediate or susceptible to a specific antibiotic are indicated in grey, light grey, or white, respectively. AM: ampicillin; AMC: amoxicillin-clavulanic acid; CF: cephalothin; CTX: cefotaxime; GM: gentamicin; K: kanamycin; S: streptomycin; TE: tetracycline; CIP: ciprofloxacin; NA: nalidixic acid; SMZ: sulfamethoxazole; TMP: trimethoprim; C: chloramphenicol. m: male; f: female; nd: no data.

were found, amounting to 19 % of the analyzed strains. In the age group comprising the 6 to 14 year-olds, 3 strains were found (14.3 %). The majority (11) of the cases (52.4 %) was found in the age group of patients between the ages of 15 to 65. One strain originated from a person older than 65 (4.8 % of all strains). Eleven (52.4 %) of the patients were female, 9 (42.9 %) were male and for one patient the gender was unknown.

Of all patients, 4 had a documented history of traveling abroad. Two patients belonging to the age group of 15–65 years had visited Tunisia. One patient of the same age group had traveled to Bulgaria and one infant was connected to a stay in Brazil.

One person was a carrier who was routinely checked for enteric pathogens at his work place.

Antimicrobial susceptibility

Strains exhibiting resistance to more than three classes of antibiotics were designated multidrug resistant. For the calculations of percentages, intermediate susceptibility was counted as susceptible.

All 21 strains (100 %) were resistant to sulfamethoxazole. One strain was also resistant to streptomycin and one to tetracycline. Two isolates (9.5 %) were classified multidrug resistant, with one strain revealing resistance to ampicillin and nalidixic acid and one exhibiting resistance to ampicillin, gentamycin, kanamycin and nalidixic acid (Tab. 1).

All strains were fully susceptible to the combination of the ß-lactam antibiotic amoxicillin with the ß-lactamase inhibitor clavulanic acid and all strains were fully susceptible to the 3rd-generation cephalosporin cefotaxime. None of the strains were resistant to ciprofloxacin, trimethoprim or to chloramphenicol.

From 2006 to 2010 no major shift in resistance patterns was observed.

Genetic relationship of Swiss *S.* Schwarzengrund isolates

PFGE analysis revealed 17 similar banding patterns among the 21 isolates. The dendrogram generated from Dice coefficients of similarity indicated that all isolates were related with Dice values greater than 80 %, with 11 strains (52 %) sharing 90 % similarity (Fig. 1). Four strains (19 %) shared a similarity of 100 %. Two of these indistinguishable strains were from patients with a history of travel to Tunisia and 2 had an unknown history of travel. No further connection between the patients could be established and no common food or other source of infection was identified. Samples had been obtained and submitted to the NENT from all over Switzerland.

One strain (N06-226) displayed a banding pattern that shared very high similarity to pattern JM6X01.0091, a pattern common among isolates that are disseminated internationally by chicken products from Thailand to humans in Denmark and the USA (Aarestrup et al., 2007).

Discussion

On average, the annual incidence of salmonellosis in Switzerland for the years 2006 to 2010 was 21.25/100'000

(http://www.bag.admin.ch/k_m_meldesystem/00733/00813/). From this time period, isolates submitted from medical institutions from all over Switzerland originating from 21 cases of human infections with *S.* Schwarzengrund were investigated for antibiotic resistance and for genetic relationship.

Salmonella serovar Schwarzengrund is not found among the top 20 of the most frequently serotyped *Salmonella* isolates from humans in Europe (Hendriksen et al., 2011). We found the incidences of *S.* Schwarzengrund in Switzerland during the study period, although intermittently increased, to correspond with these findings.

Over the past decade, *S.* Schwarzengrund has become one of the emerging *Salmonella* serotypes isolated from humans and poultry in Asian countries such as Thailand (Bangtrakulnonth et al., 2004) and China (Chen et al., 2010). The dissemination of multidrug resistant *S.* Schwarzengrund by contaminated food products imported from Thailand to Denmark and the United States has been described (Aarestrup et al., 2007). Similarities between one of the most common PFGE patterns of isolates from that study and an isolate from our study indicate a possible origin in Thailand and the involvement of chicken meat. However, despite the similarities of the PFGE patterns, no further geographical or temporal association between the isolates could be made. No history of travel to Thailand could be established for any of the patients, and import of poultry meat from Thailand to Switzerland was very low during the study period (Swiss Federal Customs Administration FCA; https://www.swiss-impex.admin.ch/). This may possibly account for the low incidence and low rate of multidrug resistant *S.* Schwarzengrund in Switzerland. Import trade statistics show that Brazil is one of the main exporters of poultry meat to Switzerland (Swiss Federal Customs Administration FCA; https://www.swiss-impex.admin.ch/). Interestingly, a microbiological assessment of carcasses from a poultry processing plant in Brazil revealed a high prevalence of *S.* Schwarzengrund (Santos et al., 2011). However, to our knowledge, no PFGE data was available for comparison.

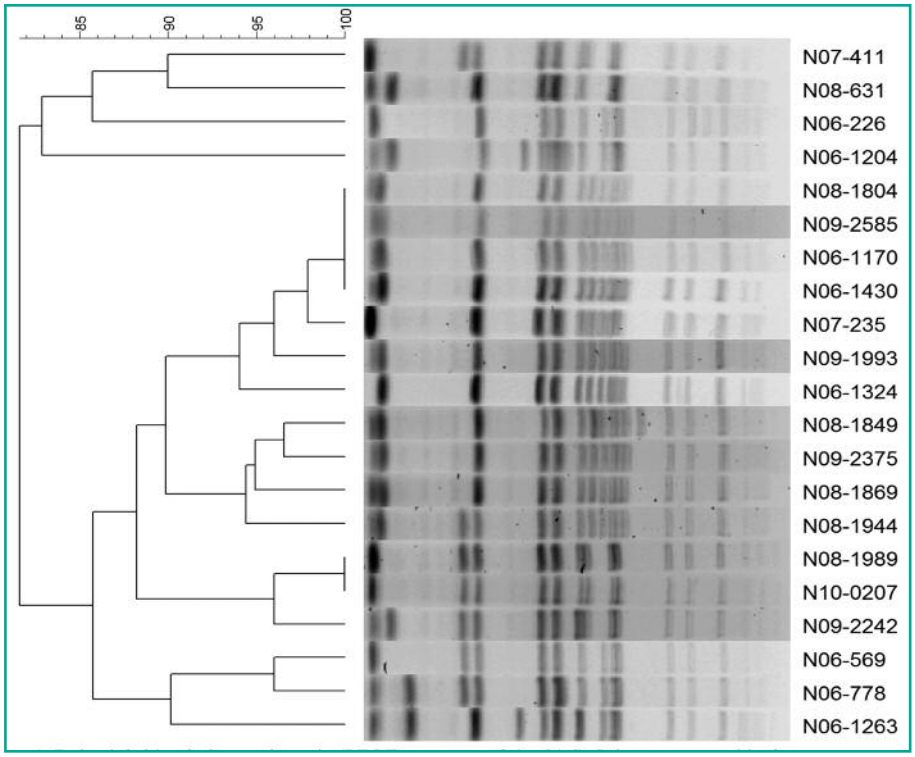

FIGURE 1: *Dendrogram showing the PFGE XbaI-patterns of the 21 S. Schwarzengrund strains.*

The emergence of multidrug resistance in pathogenic as well as commensal bacteria in general and in *Salmonella* spp. in particular is an increasing global problem (van den Bogaard et al., 2000; Threlfall et al., 2000). Resistance to nalidixic acid in salmonellae is of great concern, as it has been reported to be an indicator for reduced susceptibility to one of the first-line antimicrobials, ciprofloxacin, resulting in possible treatment failure (Aarestrup et al., 2003; Crump et al., 2003). From our collection of strains, 9.5 % (isolates N06-1204 and N08-631) were resistant to nalidixic acid, resulting in a resistance rate lower than described for other countries (Frank et al., 2007).

Reports on the emergence of extended-spectrum ß-lactam resistant salmonellae give rise to concern (Coque et al., 2008). Genes encoding for extended-spectrum ß-lactamases, predominantly bla_{TEM}, bla_{SHV} and bla_{CTX-M} variants have been detected in various *Salmonella* serovars isolated from humans as well as from poultry and poultry products (Hasman et al., 2005). However, all the *S.* Schwarzengrund isolates in this study were susceptible to cefotaxime. So far, resistance to extended-spectrum ß-lactam antibiotics is not yet an issue in *Salmonella* Schwarzengrund isolates in Switzerland.

Resistance to sulfamethoxazole is characteristic of class 1 integrons (Fluit et al., 1999). Common among Enterobacteriaceae, they constitute an important source for the evolution and dissemination of antibiotic resistance genes across the bacterial species by virtue of their ability to acquire, collect and rearrage gene cassettes (Gillings, 2014). Integrons have been detected in *S.* Schwarzengrund isolated from chicken meat from Tunisia (Soufi et al., 2012), and chicken and pig meat from Taiwan (Chen et al., 2010). The fact that all the strains in this study were resistant to sulfamethoxazole suggests the presence of integrons in these isolates. Integrons associated with resistance genes are often also associated with transposons or plasmids and are thus of major importance in the spread of antibiotic resistance genes (Gillings).

Although multidrug-resistance was detected at a comparatively low rate in our study, ongoing attention should be paid to the presence of antibiotic resistance in *Salmonella* spp. in order to monitor the global spread of antimicrobial resistance at regional and local levels and to minimize the risks of treatment failures of infectious diseases in the future (O'Brien, 1999).

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Conflict of interest

The authors declare no conflict of interest, which could influence the content or opinions presented in the manuscript.

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Address of corresponding author:
Prof. Dr. Roger Stephan
Institute for Food Safety and Hygiene,
National Centre for Enteropathogenic Bacteria and Listeria, University of Zurich,
Winterthurerstr. 272,
8057 Zurich, Switzerland
stephanr@fsafety.uzh.ch