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Salmonella in Poultry flocks and Humans – *S. enterica* subspecies *enterica* serovar Enteritidis in the history

Salmonellen in Geflügelbeständen und beim Menschen – S. enterica subspecies enterica serovar Enteritidis in der Vergangenheit

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Summary

After importing of breeder lines for laying flocks from Canada into the former GDR in 1966 the egg industry in this country was completely isolated from that in Western Germany or other Western European countries until opening the border in Germany in 1989. Because of this isolation from other countries, an analysis of the clonal diversity of *Salmonella (S.)* Enteritidis isolates originated from humans, chickens and food in the former GDR during the 1980s would provide a unique opportunity to obtain new insight into factors that may have triggered the *S.* Enteritidis epidemic.
While isolates had previously been typed by the phage typing scheme of Lalko and Laszlo we applied for the first time the extended phage typing scheme by Ward for the retrospective analysis of the S. Enteritidis strains. Furthermore, isolates of phage type (PT) 4/6 (Ward / Lalko and Laszlo) from different livestocks were investigated by ribotyping. Although in total the PT4/6 dominated between 1986 and 1989 in poultry, other phage types have prevailed in the early 1980s and represented a considerable fraction of isolates until 1989. For instance, PT8/7 was isolated from one large layer farm (district Halle) from 1988 until 1989. During that time in another farm (district Cottbus) only PT8/7 was detected too. PT4/6 was isolated from neither of these two laying hen farms. The strains of PT4/6 could be distinguished by ribotyping in 19 different subtypes. The strains from the northern farms were distinct from those isolated in the southern regions.
As farms which were harbouring either PT4/6 or PT8/7 had obtained laying hens from the same sources (breeder lines in Deersheim and Spreenhagen) it is highly probable that *S.* Enteritidis infection was acquired from the environment at each individual farm. This conclusion is also supported by the presence of different PT4/6 ribotypes in different farms. The presence of different phage types or PT4/6 ribotypes at different farms of laying hens suggests that in each case the *S.* Enteritidis strains present in the environment were able to enter chicken flocks.

Keywords: *Salmonella* Enteritidis, epidemiology, poultry, outbreaks, phage types, ribotypes

Zusammenfassung

Nach dem Import der Zuchtlinien für Legehennen aus Kanada im Jahr 1966 war die Eierindustrie in der ehemaligen DDR bis zur Grenzöffnung 1989 von Westdeutschland und anderen westeuropäischen Ländern vollständig isoliert. Aufgrund dieser zu anderen Ländern isolierten Situation bot sich die einzigartige Möglichkeit, eine Analyse der klonalen Verschiedenartigkeit der *Salmonella (S.)*-Enteritis-Isolate von Menschen, Hühnern und Lebensmitteln aus der früheren DDR durchzuführen und somit neue Informationen zur *S.*-Enteritidis Epidemiologie zu erhalten.
Während früher die Isolate nach dem Lysotypie-Schema nach Lalko und Laszlo untersucht worden waren, wurden in dieser retrospektiven Analyse die *S.*-Enteritidis-Stämme nach dem 1999 erweiterten Schema nach Ward typisiert. Darüber hinaus wurden die Stämme des Lysotyps 4/6 mittels Ribotypie untersucht. Obwohl der Lysotyp 4/6 (Ward/Lalko and Laszlo) von 1986-1989 im Geflügel dominierte, kamen andere Lysotypen in den frühen 80er Jahren vor und machten einen gewissen Anteil bis 1989 aus. So wurde im KIM Gutenberg (Bezirk Halle) von Juli 1988 bis Dezember 1989 der Lysotyp 8/7 isoliert. In dieser Zeit wurde auch im KIM Roggosen (Bezirk Cottbus) nur der Lysotyp 8/7 identifiziert. Lysotyp 4/6 hingegen wurde in diesen beiden Legehennenbeständen während der ganzen Zeit nicht isoliert. Die Stämme des Lysotyps 4/6 konnten durch die Ribotypie in 17 verschiedene Subtypen unterteilt werden. Die Stämme aus den nördlichen KIM Betrieben unterschieden sich von den südlichen KIM Betrieben. Da in den Legehennenbeständen, die von der gleichen Zuchtlinie (Deersheim und Spreenhagen) stammten, entweder Stämme des Lysotyps 4/6 oder 8/7 vorkamen, mussten die *S.*-Enteritidis-Infektionen aus der entsprechenden Umwelt der einzelnen Farmen aquiriert worden sein. Diese Schlussfolgerung wird auch durch das Vorkommen von verschiedenen Ribotypen des Lysotyps 4/6 in verschiedenen Beständen unterstützt. Aufgrund des Nachweises von unterschiedlichen Lysotypen oder Ribotypen von LT4/6 in verschieden Legehennenbeständen kann vermutet werden, dass der Eintrag der *S.*-Enteritidis-Stämme in jedem Falle aus der Umwelt in die Bestände erfolgte.

Schlüsselwörter: *Salmonella* Enteritidis, Epidemiology, Geflügel, Ausbrüche, Lysotyp, Ribotyp

Introduction

In the 1980s, public health laboratories in Europe and the Americas reported a dramatic increase in the number of human *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) infections (Rodrigue et al., 1990). During the time from 1986–1988 the percentage of notifiable gastrointestinal diseases increased to 12 % and was the group second in importance next to the acute respiratory diseases in East Germany (Rasch and Dittmann, 1989). The percentage of *S.* Enteritidis increased in 1987 to 34 % of all *Salmonella* isolates starting with 4 % in 1985 over 21 % in 1986 (Böhme et al. 1989). In Germany, *S.* Enteritidis became the serovar of *Salmonella* most frequently isolated from humans in 1986 and the highest incidence of human cases was reported in 1992 with an incidence of 242.4 cases/100.000 inhabitants (Tschäpe et al., 1999). During this *S.* Enteritidis pandemic, the majority of outbreaks in Europe and the Americas were traced back to foods containing raw or undercooked chicken eggs (Louis et al., 1988; Cowden et al., 1989). Surveillance performed by the Enter-net National Reference Laboratories since 1993 has shown that *S.* Enteritidis continues to be the predominant serovar of *Salmonella* in Western Europe. In Germany, *S.* Enteritidis accounted for 55.4 % and 60.6 % of *Salmonella* serovars isolated from humans in 1997 and 2005, respectively. In 2005, the National Reference Center for *Salmonella* and other Enteric Pathogens in Wernigerode identified 49 *S.* Enteritidis outbreaks in Germany (Anonymous, 2006). *S.* Enteritidis phage typing has proven to be a valuable tool for outbreak analysis and for following the spread of individual clones during the current epidemic. Analysis of epidemic isolates by phage typing demonstrated that phage type (PT) 4 was responsible for the dramatic increase of human S. Enteritidis cases during the 1980s in several Western European countries. Based on these observations it has been proposed that the *S.* Enteritidis epidemic in Europe was triggered by the emergence of a more virulent clone, represented by epidemic PT4 isolates. Data from the Enter-net *Salmonella* database indicate a recent decline in the prevalence of PT4 in Europe. In 1998, PT4 caused 61.8 % of the 21,630 reported human *Salmonella* Enteritidis cases, whereas in 2003 the share of PT4 declined to 32.1 % of the registered 8,794 *Salmonella* Enteritidis cases. During that time the number of human *S.* Enteritidis isolates belonging to other phage types increased considerably (Fisher et al., 2004).

TABLE 1: *Percentage of Salmonella Enteritidis phage types in Eastern Germany between 1986 and 1989*[1].

Phage type[2]	1986	1987	1988	1989
6	78.7	77.3	80.2	58.3
7	17.1	17.7	17.0	32.0
1	2.2	1.0	1.6	8.2
7a	0	0	0	0.1
other	2.0	4.0	1.1	1.2
Number of strains phage typed	187	322	1.404	1.902

1) All strains were isolated from human, chicken, food or environment and sent from the Hygiene-Institut or the Bezirksinstitut für Veterinärwesen to the National Reference Centre for phage typing.

2) Lalko/Laszlo phage typing scheme. Since 1993 we used the Ward scheme additionally of phage types isolated from a unique outbreak after more than 15 years of storage.

TABLE 2: *Retrospective examination of different outbreak strains by phage typing within a unique outbreak after 15 years of storage.*

Source[1]	Outbreak/ Place	Time of outbreak month/year	Number of patient strains	Phage type [2] Ward/Lalko and Laszlo
HI Gera	Stadtroda	5/86	11	4b/6
HI Frankfurt/ Oder	Frankfurt/Oder Storkow	2/88	11	4b/6
HI Berlin	Berlin	6/88	14	4b/6
HI Berlin	Berlin	6/88	2	4/6
HI Dresden	Dresden	7/88	5	4b/6
HI Bautzen	Bautzen	9/89	2	4/6
HI Leipzig	Leipzig/Grimmen	8/88	8	4/6
HI Leipzig	Leipzig/Altenburg	8/88	4	4/6
HI Suhl	Suhl/Gotha	9/89	6	4/6

1) HI – Hygiene-Institut.

2) The second edition of the Ward scheme allows differentiation between phage type 4 and phage type 4b.

TABLE 3: *Occurrence of different S. Enteritidis PT 4/6 ribotypes in outbreaks and associated food in former Eastern Germany.*

Ribotype	Year	Number of strains	Human origin	Associated food
1	1986	3	Erfurt	Salmon-herring pastry and raw egg
7	1988	3	Rostock	Lemon cake, egg
1a	1988	2	Dessau	Raw egg
1	1989	2	Leipzig	Ice cream
1	1989	2	Gotha	Lemon cream
7	1989	2	Brandenburg	Cake

TABLE 4: *Occurrence of different S. Enteritidis PT 4/6 ribotypes from chicken farms.*

Source	Year	Ribotype	Origin	Farm
BIV[1] Jena	1986	1	Egg	KIM[2] Wandersleben
BIV Meiningen	1988	1a	Chicken	Dillstädt
BIV Frankfurt/Oder	1988	6	Chicken	KIM Storkow
BIV Karl-Marx-Stadt	1988	2	Chicken	KIM Neukirchen
BIV Neubrandenburg	1989	8	Chicken	VEG[3] Diemitz
BIV Potsdam	1989	8	Chicken	KIM Königswusterhausen
BIV Rostock	1989	8	Chicken	KIM Neubuckow

1) BIV – Bezirksinstitut für Veterinärwesen.

2) KIM – Kombinat Industrielle Mast.

3) VEG - Volkseigenes Gut.

In this study a reinvestigation of *S.* Enteritidis strains of different origin, isolated two decades ago in the former Eastern part of Germany (GDR, German Democratic Republic) was carried out to gain more detailed information on ways of infection, spread and establishment of specific phage types of this important serovar.

Material and Methods

Strains

3,815 *S.* Enteritidis strains were phage typed during 1986–1989 and stored at the National Reference Centre in the former Eastern Germany (German Democratic Republic, GDR) from 1986–1989 (Tab. 1). All these *S.* Enteritidis strains, reinvestigated in this study, were isolated from chicken, eggs, food and human beings. A total of 41 strains belonging to PT4b/6 from outbreaks were investigated to establish the stability of phage typing after storage for 15 years, because the isolates were stored on Dorset-Agar at 8 °C and recultured on Endo-Agar plates. The original serological identification of O9 and Hg,m with anti *Salmonella* sera was confirmed before investigation. Furthermore, 24 strains of S. Enteritidis phage type 4/6 were investigated by ribotyping.

Phage typing

The routine phage typing of *S.* Enteritidis was performed according to the typing system of Ward/Lalko and Laszlo, e.g. PT4/6, phage type 4 according to Ward and 6 according to Lalko and Laszlo (Laszlo et al., 1985; Rabsch, 1996; Ward et al., 1987).

Ribotyping

DNA isolation was performed according to "Standard procedures in Molecular Biology" (Ausubel et al., 1997). DNA was digested with the restriction enzymes *PstI* and *SphI* and fractionated by electrophoresis (Liebana et al., 2002). DNA fragments were transferred to a positively charged nylon membrane (Boehringer) by vacuum blotting as recommended by the supplier (Pharmacia) and fixed to the membrane by crosslinking (GS Gene Linker™, Bio-Rad). The digoxigenin-11-dUTP-labelled c-DNA probe was synthesized from 16S and 23S ribosomal RNA from *E. coli* using AMV reverse transcriptase and the labeling was carried out according to the Boehringer protocol. The designation of ribotypes was made arbitrarily by lane numbering, the most frequent type was given number 1. Until now strains of PT 4/6 can be subtyped in 19 different ribotypes. In this study 8 ribotypes were observed.

Results

The *Salmonella* Enteritidis strains which were collected between 1986–1989 from outbreaks by different human and veterinary institutes were sent to the National Reference Centre for phage typing to investigate the epidemiological context. The dominant phage type of *Salmonella* Enteritidis isolated from

human beings, different animal species, food or environment in Eastern Germany was PT 6 (Lalko/Laszlo) but other phage types as PT8 and PT1 were also detected. The percentages of these different phage types are presented in Table 1. Since 1993 beside the phage typing scheme from Lalko/Laszlo we also used the typing scheme developed by Linda Ward. A retrospective examination of different outbreak strains, which were originally isolated from different regions in the former GDR, after storage of the cultures for 15 years reveals the uniformity of phage types of *Salmonella* Enteritidis strains. In 9 outbreak strains all strains from human and food of the single outbreak had the identical phage type (Table 2). Furthermore, using the Ward phage typing set a differentiation between PT4 and PT4b outbreaks was possible. By using the extended phage typing scheme we could identify three districts (KIM Hermsdorf, KIM Bernau and KIM Storkow) where *S.* Enteritidis PT4b occurred in humans and chickens. In other districts (KIM Neubuckow, KIM Wandersleben and KIM Neukirchen) the classic PT4/6 dominated. The large outbreaks in the districts Erfurt, Dresden and Berlin in October 1986 were caused by strains isolated from salmon-herring pastry delivered to these regions, all isolates belong to PT4/6. In 1989, isolates of *S.* Enteritidis PT 1/1 were frequently found in the district Magdeburg and the KIM Möckern (Fig. 3).

Using ribotyping PT4/6 isolates from different sources could be further differentiated. We applied the subtyping method by *PstI/SphI* double digest to *S.* Enteritidis PT4/6 isolates. The reinvestigation of *S.* Enteritidis phage type 4/6 strains from human outbreaks and associated food could be grouped by ribotyping in different subtypes (Table 3). Furthermore the isolates from different veterinary institutes sent to the National Reference Center belonged to different ribotypes as well. The strains from the northern farms (KIM Neubuckow and VEG Diemitz) were distinguished from those isolated in the southern GDR, e. g. KIM Wandersleben and KIM Neukirchen (Table 4). Three strains of PT4/6 isolated from an egg (KIM Wandersleben) belong to ribotype 5, another egg-isolate (KIM Neubuckow) to ribotype 11 and one human derived strain (HI Erfurt) to ribotype 3.

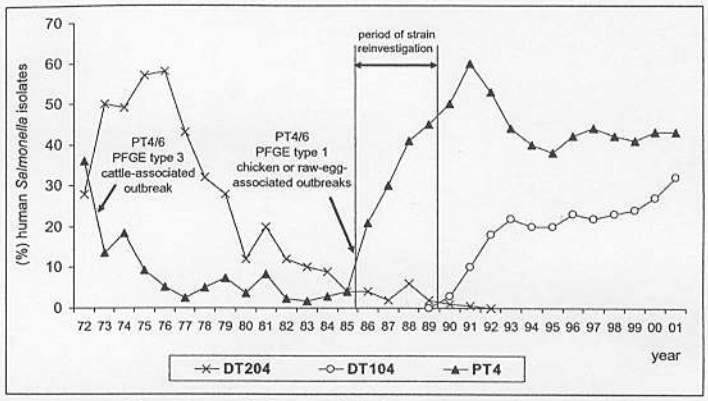

FIGURE 1: *Dominant Salmonella clones in the former Eastern Germany since 1990 the Neue Bundeslaender and Berlin (PT = phage type; DT = definitive type).*

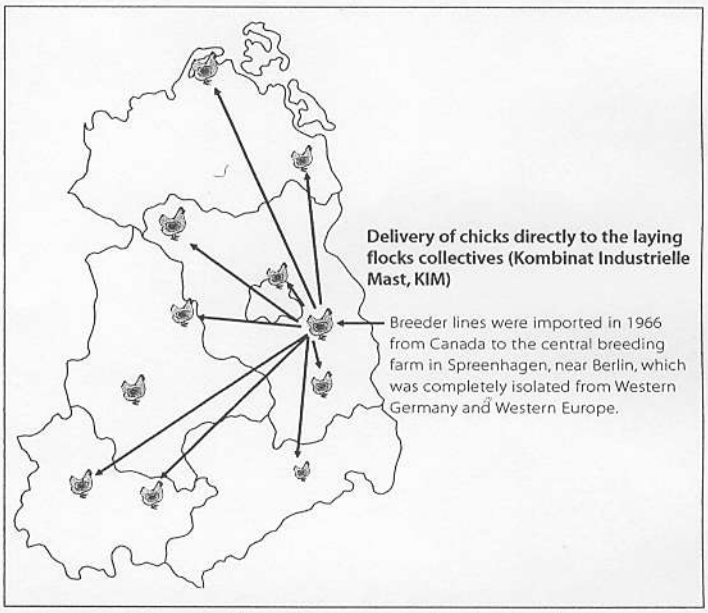

FIGURE 2: *Organisation of the egg production in the former Eastern Germany 1986–1989.*

Discussion

Prior to 1984, *S.* Enteritidis was isolated only sporadically from humans in the former GDR. There was a brief increase in the incidence of human *S.* Enteritidis cases in 1972, which was traced back to the consumption of contaminated beef from calves carrying the organism. This sporadic increase of *S.* Enteritidis cases attracted little attention since it was overshadowed by a more dramatic increase in human infections with *S. enterica* subsp. *enterica* serovar Typhimurium phage type DT204 that could be also linked to cattle (Rabsch et al., 2001). By 1973 *S.* Enteritidis had disappeared again from the epidemiological landscape and continued to be isolated only rarely from human beings in Eastern Germany until 1984 (Fig. 1).

However, there is evidence that *S.* Enteritidis became associated with chicken eggs before the 1980s in Western Germany. For instance, in Western Germany the number of human *S.* Enteritidis cases was monitored between 1973 and 1982 by a National Surveillance Programme (Zentrales Überwachungsprogramm *Salmonella*, ZÜPSALM). Although *S.* Enteritidis caused only a relatively small number of outbreaks in Western Germany during the 1970s, most of them could be linked to foods containing raw or undercooked chicken eggs (Rabsch et al., 2001). In the district Erfurt in southern Eastern Germany a strong increase of salmonellosis was recorded between 1986 and 1987, which was caused by *S.* Enteritidis in 70 % of all human cases and associated with the consumption of eggs or raw egg products (Ullmann and Scholtze, 1989). Complex control measures in the egg production were initiated to minimize human infection (Müller and Körber, 1991). Infected breeder flocks were identified to be the primary source of vertical *Salmonella* transmission to the progeny. This route is followed by horizontal often self maintaining infection cycles in the flocks. Environmental *Salmonella* organisms may also enter such cycles (Heider, 1989).

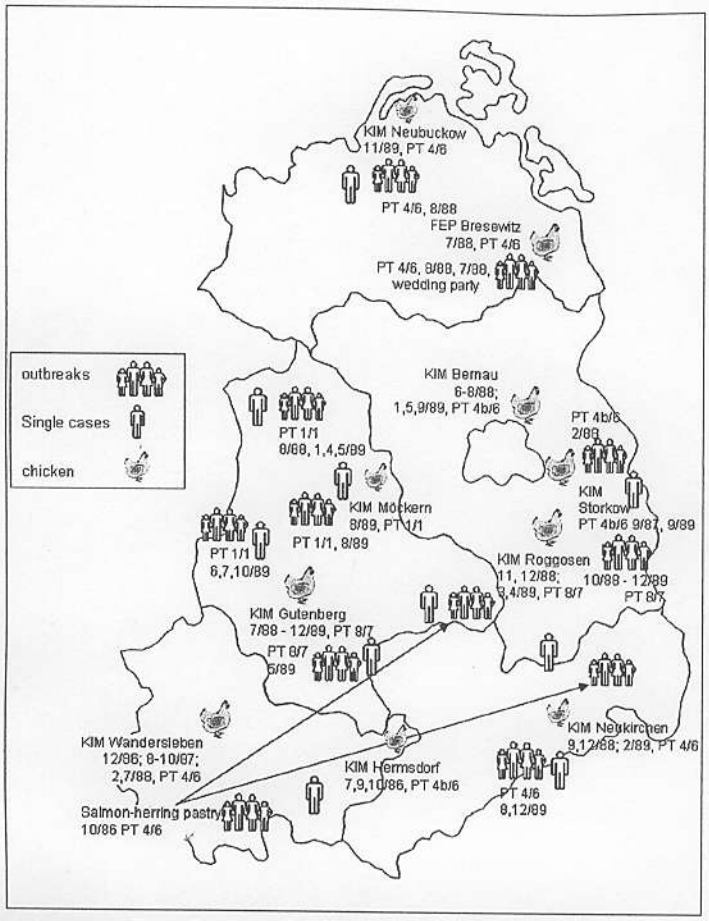

FIGURE 3: *Distribution of different S. Enteritidis phage types in Eastern Germany from 1986–1989 (4/89 = April/1989; PT4/6 = 4 Ward scheme/ Lalko and Laszlo scheme).*

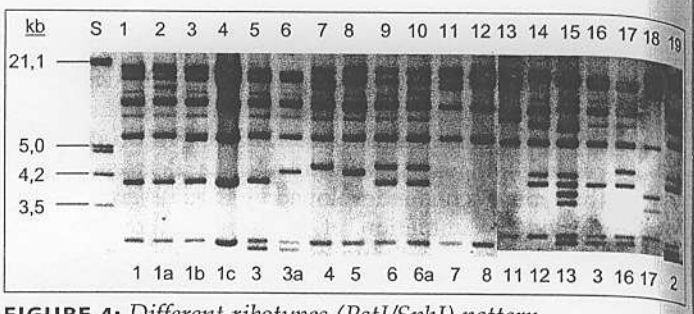

FIGURE 4: *Different ribotypes (PstI/SphI) pattern of S. Enteritidis.*

We decided to reinvestigate the *S.* Enteritidis strains isolated two decades ago in the GDR, because the poultry industry in this country ideally lends itself to an analysis of the *S.* Enteritidis epidemic for the following reasons:

1. After breeder lines for laying flocks had been imported from Canada in 1966 the egg industry in the GDR was completely isolated from that in Western Germany or other Western European countries until opening of the borders to Western Germany in 1989. This closed system represents an unique opportunity to investigate the origin of the *S.* Enteritidis epidemic.
2. In the former GDR 16.4 million inhabitants lived in 15 regional districts. The laying hen population in this country composed of 24.4 million birds, thus representing a large sample size for epidemiological studies.
3. From a central breeding farm (Spreenhagen, near Berlin), the chicks were delivered directly to the laying hen farms (Kombinat Industrielle Mast KIM). The known trade routes of chickens enabled us to analyse whether *S.* Enteritidis was introduced at the layer farms locally or by acquiring infected birds from the central breeding farm (Fig. 2).

Because of the findings in other countries, we assumed that an analysis of the clonal diversity of *S.* Enteritidis isolates originated from humans, chickens and food in the former GDR during the 1980s would provide an unique opportunity to obtain new insight into factors that may have triggered the *S.* Enteritidis epidemic. While isolates had previously been typed by the phage typing scheme of Lalko and Laszlo we applied for the first time the extended phage typing scheme by Ward which was introduced in our lab in 1999 for retrospective analysis of these strains. Different phage types (PT6, PT1, PT7, scheme Lalko and Laszlo) of *S.* Enteritidis, isolated from a laying flock in Wandersleben/Thuringia from June 1988–July 1989 could be differentiated (Ludwig and Calsow, 1992). In this laying flock neither a decrease in egg production nor any evidence of clinical signs were observed (Müller and Körber, 1991).

Although in total the Ward phage type 4/6 dominated between 1986 and 1989 in poultry, other phage types prevailed in the early 80s and represented a considerable fraction of isolates until 1989. Large outbreaks in the districts Erfurt, Dresden and Berlin were caused by strains typed as *S.* Enteritidis PT4/6. Some of these outbreaks were caused by salmon-herring pastry with 275 registered cases involved in October 1986 (Ullmann and Scholtze, 1989). Furthermore, PT8/7 was isolated from the farm Gutenberg, district Halle and the farm Roggosen, district Cottbus between July 1988 and December 1989 (Fig.3). PT4/6 was isolated from neither of these two farms.

Using ribotyping PT4/6 isolates from different sources could be further differentiated. We applied the subtyping method by *PstI/SphI* double digest to *S.* Enteritidis PT4/6 isolates (Landeras and Mendoza, 1998; Liebana et al., 2001; Clifford et al., 2003). The strains of phage type PT4/6 could be distinguished by ribotyping in numerous different subtypes (Fig. 4). The strains from the northern farms (KIM Neubuckow) were distinct from those isolated in the southern GDR, e. g KIM Neukirchen (Table 3).

As farms which were harbouring PT4/6, PT4b/6 or PT8/7 either, had obtained laying hens from the same sources (breeder lines in Deersheim and Spreenhagen) it is highly probable that the *S.* Enteritidis infection was acquired from the environment at each individual farm. This conclusion is also supported by the presence of different PT4/6 ribotypes in diverse farms. The presence of different phage types or PT4/6 ribotypes at different farms of laying hens suggests that in each case the *S.* Enteritidis strains present in the environment (i.e. in rodent or vertebrate populations) were able to enter chicken flocks.

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