Molecular marker analysis of *Salmonella typhimurium* from surface waters, humans, and animals

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**Abstract.** *Salmonella* contamination of North Sea water was detected for the first time in 1988 in Germany during routine examinations of bathing areas. Since then, subsequent isolations along the coast have been reported regularly. To define the source of contamination, strains isolated from seawater and rivers were studied by molecular marker methods. Their properties were compared with those of strains originating from possible sources of contamination such as humans, cattle, and sewage treatment plant water. Plasmid profile analysis of whole bacterial populations and the determination of antibiotic resistance patterns demonstrated, that contamination through the surrounding cattle industry could be excluded. Cattle isolates belonged to a widespread clone of phage type 204c which was multiresistant and exhibited an unique plasmid pattern which was never found in sea water isolates. Outer membrane protein and lipopolysaccharide analysis failed to demonstrate differences among the *Salmonella* populations and proved in this case insufficient for molecular marker discrimination.

**Key words:** Multi-resistance, Plasmid typing, Phage type 204c, *Salmonella typhimurium*, Sewage

**Introduction**

In many countries the reported cases of salmonellosis have increased steadily during the last few years [6, 33, 42, 44]. Since the numbers reported are in general substantially lower than the actual cases [22, 42], it can be concluded that *Salmonellae* are one of the main causes of foodborne diseases worldwide. Human and animal salmonellosis is generally associated with the consumption of contaminated food or feed. Farm animals, especially poultry, are frequently carriers of *Salmonella* and pass the organisms along the food chain, finally causing human infections. Spread of *Salmonellae* into the environment could occur via faecal excretion by humans and animals, especially by the disposal of slurry. This may lead to a contamination of surface waters and closes the cycle of *Salmonella* transmission by recolonization of farm animals.

Most human *Salmonella* outbreaks are related to food. However, isolation of *Salmonella* strains from polluted natural waters has been described [30], and there is evidence that salmonellosis can follow the contamination of water supplies [16, 34]. Major outbreaks, however, have not been reported.

The study described here was initiated after having obtained *Salmonella*-positive samples in routine examinations of North Sea water in 1988 [7]. This led to the closure of public beaches, according to the EC guidelines for bathing sites [2], and raised much public attention. Since then, *Salmonella* contamination has been monitored intensively along the coast of the mainland and parts of the East Frisian islands [41].

The majority of *Salmonella* isolates from North Sea water consisted of the serotype *Salmonella typhimurium* including its O5-negative variant, although a variety of other serovars (e.g. *S. mbandaka, S. infantis, S. enteritidis, S. virchow*) have been detected as well. In order to elucidate the sources of water contamination, we examined all *S. typhimurium* strains originating from suspected sources by molecular marker methods. These methods have proved to be highly efficient in other epidemiological studies [for review, see 14 and 39]. Because the area involved has a very intensive calf fattening industry, cattle discharge was considered as a possible source of contamination. Furthermore, sewage was also suspected as a candidate responsible for the contamination of the surrounding surface waters. *Salmonellae* from human sources might reach surface waters either by waste water treatment plants, the disposal of the contents of faecal tanks of ferry boats into the sea, or even direct contamination by bathing and camping sites, etc. Consequently, the properties of the surface water isolates were
compared with strains originating from cattle, from human patients, and from sewage treatment plant water of the surrounding area. Strain differentiation was achieved by determining plasmid profiles and antibiotic resistance patterns.

Materials and methods

Bacterial strains. The Salmonella isolates obtained from water include isolates from the North Sea coast (mainland and islands), from the river Ems, lakes with beaches, ponds, and from sewage treatment plants. Salmonella isolates of human and animal origin were sampled from human patients and cattle. A total of 606 Salmonella typhimurium strains isolated in northern Germany were examined. 119 strains consisted of surface water isolates originating from North Sea water, ponds, lakes, and rivers (n = 36). 76 strains came from sewage treatment plants. Their molecular epidemiological markers were compared with those of 230 strains isolated from humans and 181 strains from infected cattle in the surrounding area. Isolation and serotyping were done as described [2, 11]. Comparison of the plasmid distribution in S. typhimurium always included the 05 negative variant copenhagen as well. Escherichia coli C600 was used to propagate the probe carrying plasmid (pRQ51) for the hybridization procedures [29].

Isolation and analysis of plasmid DNA. Plasmid DNA from Salmonella was prepared by modifying the method of Kado & Liu [19]. 1.5 ml of a Salmonella overnight culture (37 °C, not shaken) in L-broth were pelleted by centrifugation (4 min, Eppendorf centrifuge). The pellet was resuspended in 20 μl 50 mM Tris-HCl/1 mM EDTA pH 8 and after adding 100 μl of lysis solution the tubes were incubated at 58 °C for 25 min. The solution was carefully mixed with phenol/chloroform (1:1, v/v) and the phases were separated by centrifugation (15 min). The aqueous phase was transferred to a new tube and 25 μl were used for electrophoresis on 0.8% agarose gels, stained with ethidium bromide and visualized under UV light. Molecular weights were determined by comparing the relative mobilities of the plasmid DNAs with those of the standards listed in reference 15.

Colony hybridization. The DNA probe consisted of a 3.6 kb HindIII-fragment of plasmid pRQ51 [29] carrying sequences of the Salmonella choleraesuis virulence plasmid, isolated by electroelution [27]. E. coli plasmid pRQ51 was prepared with the alkaline lysis method of Maniatis [27]. Bacterial strains were subjected to hybridization by the procedure of Maas [26].

Antibiotic susceptibility testing. The antibiotic susceptibility of the Salmonella strains was determined by the agar diffusion assay. The antimicrobial discs used contained: tetracycline (te) 10 μg, ampicillin (ap) 10 μg, chloramphenicol (cm) 30 μg, kanamycin (kn) 30 μg, trimethoprim/sulfamethoxazole (su) 25 μg (= 1.25 μg/23.75 μg), gentamicin (gn) 10 μg, enrofloxacin (en) 5 μg.

Isolation of outer membrane proteins. The outer membrane proteins were prepared as previously described [1].

Extraction and purification of lipopolysaccharides (LPS). The LPS of the bacterial strains were analyzed as described [17]. The purified material was separated by SDS-PAGE and visualized by silver staining [43].

Phage typing. Phage typing was kindly performed by Linda Ward, Central Public Health Laboratory Service, Colindale, London, UK.

Results

Resistance to antibiotics. The strains displayed clear differences in their antibiotic resistance profiles with respect to their origin (Table 1). Most of the human (58.3%) and the different water isolates (surface waters 70.6%, sewage treatment plants 85.5%) were antibiotic-sensitive. In contrast, nearly all (97.8%) of the cattle-derived strains showed drug resistance. The majority of resistant strains in surface waters and humans displayed resistance to one antibiotic only (mainly tetracycline), whereas monoresistance was absent in cattle isolates and found to only a minor extent (6.6%) in sewage treatment plant water isolates. In addition, the proportion of multiple resistances (resistances to more than one antimicrobial agent) was highest in cattle (97.8%) followed by human (16.9%), sewage treatment plant (7.9%) and surface water (5.9%) isolates.

The different patterns of resistance to the antimicrobial agents used, and their distribution among

| Table 1. Antibiotic resistance in Salmonella typhimurium isolated from various sources (in %) |
|------------------------------------------------------|-------|-------|-------|-------|
| No. of resistance phenotypes | No. of strains | | | |
| | Surface waters | Sewage treatment plant | Humans | Cattle |
| | (n = 119) | (n = 76) | (n = 230) | (n = 181) |
| 0 (susceptible) | 70.6 | 85.5 | 58.3 | 2.2 |
| 1 resistance | 23.5 | 6.6 | 24.8 | 0.0 |
| Multiple resistance | 5.9 | 7.9 | 16.9 | 97.8 |