Identification of Fecal Input Sites in Spring Water by Selection and Genotyping of Multiresistant *Escherichia coli*[∇]

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The localization of fecal input sites is important for water quality management. For this purpose, we have developed a new approach based on a three-step procedure, including a preparatory phase, the screening of multiresistant bacteria using selective agar plates, and a typing phase where selected *Escherichia coli* isolates are characterized by antibiotic resistance profiles and molecular fingerprinting techniques (pulsed-field gel electrophoresis [PFGE]). These two well-known source tracking methods were combined in order to reduce cost and effort. This approach was successfully applied under field conditions in a study area located in the north-western part of Switzerland. *E. coli* isolates from spring water and surface water samples collected in this area were screened with selective agar plates. In this way, 21 different groups, each consisting of strains with the same pattern of antibiotic resistance, were found. Of these, four groups were further analyzed using PFGE. Strains with identical PFGE profiles were detected repeatedly, demonstrating the suitability of this method for the localization of fecal input sites over an extended period of time. Identical PFGE patterns of strains detected in water from two different springs were also found in the stream flowing through the study area. These results demonstrated the applicability of the new approach for the examination of incidents of fecal contamination in drinking water. The advantages of the described approach over genotyping methods currently being used to identify sources of fecal contaminants are a reduction in time, costs, and the effort required. Identical isolates could be identified without the construction of large libraries.

Escherichia coli is commonly used for the microbiological quality assessment of drinking water (11). Its characteristics of being easily detectable, not normally pathogenic to humans and present in higher concentrations than pathogens makes this organism a good candidate as a general indicator of fecal pollution (27). *E. coli* is found in the intestines of warm-blooded animals and has been used in different microbial source tracking (MST) approaches to discriminate between human and animal fecal contamination. Different phenotyping methods, such as biochemical tests, carbon utilization profiles, fatty acid methyl ester, and antibiotic resistance analysis, have been used in MST studies employing *E. coli* as an indicator (1, 10, 23, 30). Analyses of *E. coli* using genotypic methods such as ribotyping, various PCR methods, pulsed-field gel electrophoresis (PFGE), and O serogrouping were used for MST purposes as well (6, 19, 24, 25, 28). Most of these methods are library dependent, requiring a host origin database. The genetic heterogeneity and temporal and spatial variability of *E. coli* populations have been assessed in different studies (2, 15, 16, 18, 27). Scott et al. (27) draw attention to the genetic heterogeneity by reviewing different phenotypic and genotypic methods. In two studies, *E. coli* populations were analyzed over a certain time period by ribotyping in one study (16) and antibiotic resistance analysis and ribotyping in the other (2).

Both studies concluded that the assessed temporal variability of *E. coli* populations may limit their use as MST indicators and that large databases are needed.

In another study, the importance of local and temporally specific libraries, even in very small study areas, was highlighted (18). The need for a large number of environmental and host origin isolates has also been illustrated in a study matching environmental isolates (15).

The localization of fecal input sites in water resources is of great interest and has the potential to contribute to more cost- and time-effective remediation measures. In order to reduce the cost and effort associated with currently applied methods, we tested a new approach, which makes use of the aforementioned variability within *E. coli*. It is based on a three-step procedure that includes two known source tracking methods. In order to reduce the number of isolates for subsequent analysis, a selective plate was prepared in a first step and then used for the screening for multiresistant strains, which, finally, were typed using an antibiogram and PFGE. Using this approach, we investigated a study area in Switzerland with two springs known to be vulnerable to fecal contamination and displaying a high level of pollution, especially after rainfall (3). The objective was to localize possible fecal input sites in environmental samples from the study area.

MATERIALS AND METHODS

Study area and sampling sites. The study area was situated in the Lützel valley in the northwestern part of Switzerland, shown in Fig. 1. Two karst springs named Lützelquelle (LQ) and Kächbrunnenquelle (KQ) were investigated. The springs are located close to the Lützel stream, downstream from a wastewater

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FIG. 1. Study area and sampling sites. LQ, LQ spring; KQ, KQ spring; WTP, wastewater treatment plant; LU, Lützel upstream from WTP; LD, Lützel downstream from WTP; LC, Lützel close to KQ spring. (Reproduced by permission of swisstopo [BA11001].)

treatment plant (WTP) with outlets flowing into the stream. Previous studies showed that both springs are vulnerable to fecal contamination, especially after rainfall (3). The Lützel stream is known to infiltrate the KQ spring to some extent. In contrast, the LQ spring is fed by infiltrating rainwater from a hamlet named Niederhuggerwald. The hamlet is characterized by intense dairy farming and animal husbandry and is located on a plateau to the southwest of the valley (3).

Besides the LQ and KQ springs, three surface water sampling sites along the Lützel stream were investigated. Surface water sampling sites included one site upstream (LU) and one site downstream (LD) from the WTP, close to a flowing WTP outlet. The third site was approximately 1 km further downstream from the WTP, very close to the KQ spring (LC).

Basic principle of the approach. The new approach tested in the study is based on a three-step procedure outlined in Fig. 2. In a preparatory phase (phase I), a selective plate for the screening for multiresistant strains was made. The plate was then used for a screening (phase II). In a last phase (phase III), isolates were typed by antibiotic resistance analysis and PFGE.

Phase I: preparation of agar plates to select multiresistant bacteria. A selective antibiotic plate was generated to screen for multiresistant strains. The selection of antimicrobial agents used to prepare the selective antibiotic plate known as TSA4 was based on the resistance profile of one multiresistant *E. coli* strain isolated from the LQ spring during the preliminary phase of the study. TSA4, consisting of tryptone soy agar (TSA; Oxoid AG, Pratteln, Switzerland), was supplemented by the following four antibiotic additives: streptomycin (25 µg/ml), sulfamethoxazole (150 µg/ml), tetracycline (20 µg/ml), and trimethoprim (10 µg/ml). An additional selective plate, known as TSA7 and based on another strain isolated later during the study, was also prepared. TSA7 contained the same antibiotics as TSA4, as well as three additional ones: ciprofloxacin (5 µg/ml), kanamycin sulfate (25 µg/ml), and nalidixic acid (30 µg/ml). All antimicrobials were purchased from Sigma-Aldrich GmbH (Buchs, Switzerland). The concentrations of antimicrobial agents in the plates for the selection of multiresistant strains were derived from known inhibitory concentrations (8, 21).

Phase II: sample collection, processing, and screening for multiresistant strains. Water samples were taken over a period of 7 months, namely, from March to September 2009. Three samples collected after different rainfall events, and eight dry weather samples from the two spring water sampling sites were included in the study. A rainfall event was defined as rain of more than 10 to 20 mm/h according to local weather radar. Spring water samples were collected approximately 72 h after rainfall. In addition, surface water samples were analyzed during dry weather conditions. Seven samples were collected at sampling sites upstream (LU) and 1 km downstream (LC) from the WTP, and another five samples were taken close to a flowing WTP outlet (LD).

Samples were collected in sterilized Nalgene polypropylene bottles (VWR, Dietikon, Switzerland) and were processed within 24 h after collection. Surface

I: Preparatory phase

II: Screening phase

III: Typing phase

FIG. 2. New approach based on three major phases: a preparatory phase, which resulted in a selective antibiotic plate; a screening phase to collect and analyze surface and spring water samples; and a typing phase in which isolated multiresistant *E. coli* strains were typed by antibiogram and PFGE.

water samples were taken at a depth of approximately 15 to 20 cm and away from the bank.

Defined water volumes (1,000, 100, and 10 ml for spring water and 100, 10, and 1 ml for surface water) were filtrated through Microsart CN-Filter membranes with a pore size of 0.45 µm (Sartorius Stedim Biotech GmbH, Göttingen, Germany). The filters were first incubated on TSA (Oxoid AG) for 2 h at 37°C and then transferred onto the selective TSA4 plate. As a negative control, 100 ml of distilled sterile water was filtrated and processed the same way as the water samples. Moreover, susceptible *E. coli* isolates (DSM 1103/ATCC 25922/NCIB 12210), were inoculated on the plates as a negative growth control. The plates were incubated for 16 to 20 h at 37°C; all colonies obtained from spring water samples and between 3 and 10 colonies from surface water samples were selected and inoculated on the chromogenous agar Tryptone Bile X-Glucuronide (TBX)

TABLE 1. Occurrence of multiresistant *E. coli* isolates and all *E. coli* isolates at different sampling sites

Spring or surface water sampling site	Multiresistant <i>E. coli</i> count ^a			<i>E. coli</i> count ^b		
	No. of positive samples	Median	Range	No. of positive samples ^c	Median	Range
Spring water (CFU/liter)						
Spring Lützelquelle (LQ)						
Dry weather samples	3/8	0	0–10	5/8	15	0–260
Rainfall event samples	3/3	13	8–24	3/3	3,580	1,420–13,400
Spring Kächbrunnenquelle (KQ)						
Dry weather samples	5/8	1	0–3	7/8	35	0–540
Rainfall event samples	3/3	4	0–15	3/3	14,100	2,500–76,000
Surface water (CFU/100 ml)						
Lützel upstream from WTP (LU)	6/6	7.5	1–210	6/6	285	112–21,300
Lützel downstream from WTP (LD)	4/4	155	27–260	4/4	6,550	2,010–21,700
Lützel close to KQ spring (LC)	6/6	9.5	2–440	6/6	252	140–19,900

^a That is, the cell counts after isolation with TSA4 and a further inoculation on TBX.

^b That is, the cell counts after inoculation on TBX.

^c Only 100 ml was analyzed.

agar (Oxoid AG) and incubated for 16 to 20 h at 44°C. *E. coli* strains that were β -D-glucuronidase positive were used for further typing using an antibiogram approach.

The selective TSA7 agar plate was used in addition to TSA4 for samples of one dry weather and two rainfall events. The samples were processed as described above.

Phase III: typing of isolates using antibiotic resistance analysis and PFGE. Antibiogram. A total of 173 *E. coli* isolated from TSA4 were grown on Columbia blood sheep agar (bioMérieux, Geneva, Switzerland) overnight at 37°C. Three single colonies of each isolate were then suspended in 1 ml of Mueller-Hinton broth (Oxoid AG), followed by incubation for 6 h at 37°C in a water bath. For comparative purposes, the turbidity of the suspension was adjusted with 0.5 McFarland (bioMérieux) in 1 ml of 0.9% NaCl. The isolates were streaked equally onto Mueller-Hinton agar plates (Oxoid AG).

Twelve commercially available antibiotic test discs, namely, amoxicillin-clavulanic acid (20 and 10 µg), ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), kanamycin (30 µg), nalidixic acid (30 µg), polymyxin B (300 IU), streptomycin (10 µg), sulfonamide (300 µg), tetracycline (30 µg), trimethoprim (5 µg), and trimethoprim-sulfamethoxazole (1.25 and 23.75 µg), were stamped onto the plates and incubated for 18 h at 37°C. Antibiotics active against Gram-negative bacteria that are important in human or veterinary medicine in Switzerland were chosen for screening. Sulfonamide test discs were obtained from Oxoid AG, and the remaining discs were obtained from Becton Dickinson (Allschwil, Switzerland). Inhibition zones were assessed according to international standards (CLSI/NCCLS, 2005). *E. coli* (DSM 1103/ATCC 25922/NCIB 12210) was used as a negative control strain.

Selection of isolates for further analysis using PFGE. Isolates typed with antibiogram were distributed into different groups. Each group was composed of isolates with identical resistance profiles. Groups that were composed of both isolates from spring water and isolates from surface water, displaying possible input sites, were further investigated using PFGE. Isolates from any other group were not of interest, since they did not provide information about possible fecal input sites.

PFGE. PFGE was performed according to the method of Baumgartner et al. (4), with the following modifications: agar plugs were incubated on a Thermomixer (Eppendorf, Hamburg, Germany) and washed in a Rotamix shaker (Heto Lab Equipment A/S, Alerod, Denmark). Purified DNA was digested with restriction enzymes for 5 h. Electrophoresis conditions were improved to a final switch time of 60 s and a run time of 16 h.

Electronic processing of data. The data were statistically analyzed with an SPSS Statistics 16.0 software package with a 5% significance level. The analysis of PFGE patterns was conducted with the BioNumerics version 3.5 software.

RESULTS

Occurrence of multiresistant *E. coli*. As shown in Table 1, multiresistant *E. coli* were found at all sampling sites. In spring water samples, higher median concentrations were found after

rainfall, and all samples collected after rainfall events were positive. The effect of rainfall events on the increase of multiresistant *E. coli* was statistically significant in the LQ spring but not in the KQ spring (Mann-Whitney U test, $n_1 = 8$ and $n_2 = 3$, $P = 0.024$ and $P = 0.28$).

Multiresistant *E. coli* were detected in all surface water samples. In contrast to spring water samples, surface water samples were collected only during dry weather conditions. The concentrations of multiresistant *E. coli* found in surface water samples were much higher than in spring water samples. There were no significant differences among the individual surface water sampling sites (Mann-Whitney U test, $P > 0.05$).

The concentration of multiresistant *E. coli* was much lower than the total concentration of *E. coli* (Table 1). When, in each sample, the number of *E. coli* isolated with TBX agar was compared to the number of multiresistant *E. coli* isolated with TSA4, the number of isolates was reduced by 90 to 100% in spring water samples and by 94.1 to 99.4% in surface water samples (not shown). This shows the benefits of using the TSA4 plate to reduce the number of isolates for further typing.

Typing of isolates. After 173 multiresistant *E. coli* isolates were typed through antibiogram, they could be subdivided into 21 groups. Each group was defined by an individual resistance profile. The nine groups containing strains from both surface and spring water samples were further analyzed by using PFGE. The characteristics of four groups (groups I to IV) further typed with PFGE are subsequently described in detail. Group I was composed of seven isolates with resistance to streptomycin, sulfamethoxazole, sulfonamide, tetracycline, and trimethoprim. Typing with PFGE revealed six different PFGE patterns within this group. Five isolates had different genotypes, and the remaining two isolates shared the same genotype. Group II was composed of 32 isolates with resistance to ampicillin, streptomycin, sulfamethoxazole, sulfonamide, tetracycline, and trimethoprim. Again, PFGE analysis highlighted the heterogeneity within the group, with 21 different patterns. From a total of 32 isolates, 16 represented individual genotypes, and the other 16 revealed five different genetic fingerprints between them.

Group III comprised 39 isolates with resistance to ampicil-

FIG. 3. Identical PFGE patterns of two multiresistant *E. coli* strains. One genotype, II(A), was present over 6 weeks in surface water, and another genotype, II(B), was present over 4 months in spring water. Antibiotics: AMP, ampicillin; S3, sulfonamide; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TMP, trimethoprim. Sampling sites: LC, Lützel close to KQ; LU, Lützel upstream from the wastewater treatment plant; KQ, KQ spring.

lin, chloramphenicol, ciprofloxacin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, sulfonamide, tetracycline, and trimethoprim. After PFGE analysis, six different genotypes could be differentiated, four of which were represented by individual isolates. The remaining two shared several isolates with identical PFGE patterns.

Group IV, lastly, consisted of four isolates with resistance to amoxicillin-clavulanic acid, ampicillin, ciprofloxacin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, sulfonamide, tetracycline, and trimethoprim. Two different genotypes were detected by using PFGE. Three of the four isolates were clones, and one isolate revealed a different PFGE pattern. The two groups (III and IV) contained isolates from spring and surface water with identical genotypes (see Fig. 4).

Temporal and spatial distribution of PFGE patterns. Identical PFGE patterns of multiresistant *E. coli* isolates were repeatedly detected over time. The same PFGE pattern of six multiresistant *E. coli* isolates, labeled “II(A)” in Fig. 3, revealed that, over the course of 6 weeks, a particular strain was detected repeatedly in the Lützel stream. The strain was detected on 24 March 2009 and on 7 April 2009 in the Lützel

stream upstream (LU) and downstream from the WTP (LC), on 21 April 2009 in the Lützel stream downstream from the WTP (LC), and on 5 May 2009 in the Lützel stream upstream from the WTP (LU). The identical PFGE pattern of two strains in Fig. 3, II(B), indicates repeated detection of a multiresistant *E. coli* strain in the KQ spring over a period of 4 months. This strain was detected on 21 April 2009 and on 21 July 2009 in the KQ spring.

The spatial distribution of PFGE patterns was demonstrated by the detection of identical strains at spring and surface water sampling sites. As shown in Fig. 4, a particular multiresistant *E. coli* strain, IV(D), was detected twice in the LQ spring, on 17 July 2009 and on 21 July 2009. This strain was also detected in the Lützel stream downstream from the WTP on 28 July 2009 and close to the KQ spring on the 28 July 2009 and on 18 August 2009.

Another strain found in the KQ spring was also detected at different surface water sampling sites. The pattern, referred to as III(C) in Fig. 4, indicates that this strain was found on 24 March 2009 in the Lützel stream upstream from the WTP, in

FIG. 4. Identical PFGE patterns of multiresistant *E. coli* strains in surface and spring water. A strain found in the KQ spring was also detected at different surface water sampling sites [III(C)], and a particular multiresistant *E. coli* strain [IV(D)] was detected twice in the LQ spring and in the Lützel stream. Antibiotics: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; KAN, kanamycin; NAL, nalidixic acid; S3, sulfonamide; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TMP, trimethoprim. Sampling sites: LD, Lützel downstream from the wastewater treatment plant; LC, Lützel close to KQ; LQ, LQ spring; LU, Lützel upstream from the wastewater treatment plant; KQ, KQ spring.

TABLE 2. Comparison of cell counts in water samples analyzed with chromogenic TBX agar and two selective agar plates

Water type and parameter	Presumptive <i>E. coli</i> colony counts (CFU/liter) on three different agar plates ^a		
	TBX (chromogenic)	TSA4 (four antibiotics)	TSA7 (seven antibiotics)
Spring water (<i>n</i> = 6)			
Median	280	14.5	0.5
Range	4×10^1 to 1.4×10^4	0–40	0–3
Surface water (<i>n</i> = 3)			
Median	3.9×10^4	980	60
Range	2.9×10^4 to 8×10^4	670–1,320	10–100

^a For the TSA4 and TSA7 values, the number of presumptive colonies before inoculation on TBX agar is given.

the Lützel stream close to the KQ spring, and in the KQ spring itself.

Selectivity of the antibiotic plate used for screening. Nine water samples were analyzed simultaneously with the selective TSA4 and TSA7 plates. As shown in Table 2, the addition of four or seven antibiotics to a TSA plate reduces the number of presumptive *E. coli* colonies. A reduction of at least 87% (TSA4) and 99.7% (TSA7), respectively, was observed when the number of multiresistant *E. coli* found in a particular water sample was compared to the total number of *E. coli* in the same sample (data not shown). There was a significant reduction of detected colonies with the selective antibiotic plates (Wilcoxon signed-rank test, *n* = 9, *P* = 0.008). Cell counts were lower with TSA7 than with TSA4 in both spring and surface water samples. The difference in cell counts between the plates was statistically significant (Wilcoxon signed-rank test, *n* = 9, *P* = 0.012). In addition, the number of different resistance profiles was reduced with the increase of antibiotics used to prepare the second selective plate. A total of 23 isolates grown on TSA7 agar and typed with antibiogram turned out to have the same resistance profile (data not shown). A comparison of group II and group III, described under “typing of isolates,” reveals that resistance against a higher number of antibiotics leads to a reduction of isolates with identical PFGE patterns.

DISCUSSION

Novel approach. Localization of precise input sites of fecal contamination in water resources is of great importance for water management. In the present study, we tested a new approach based on three steps: preparation, screening, and typing. A selective antibiotic plate was generated (preparation phase) and used for screening of water samples (screening phase). Based on the further characterization of *E. coli* isolates through antibiogram and PFGE (typing phase), microorganisms with identical genetic fingerprints in springs and suspected fecal input sites could be identified, thus indicating possible hydrologic connections.

E. coli is a bacterium that is well established as a general indicator for the microbiological quality of recreational and drinking water (26). Consequently, it was also proposed as an

indicator organism for use in MST. The use of library-dependent methods for the characterization of *E. coli* isolates, such as antibiotic resistance analysis or PFGE patterns for MST, however, has been questioned (28, 29).

The source allocation of *E. coli* isolates was described as difficult due to the changes of the composition of the bacterial population during the transition of *E. coli* from host to the environment, the high genetic diversity, and the excessive number of isolates required for the development of accurate libraries (7, 14, 20, 25). In the present study, we succeeded in showing that it is possible to circumvent the described variability and reduce the number of isolates to be analyzed by screening for multiresistant strains instead of typing the total number of *E. coli*.

The fact that *E. coli* populations are generally location or region specific, and therefore antibiotic-resistant profiles, as well as genetic fingerprints, differ from one site to another, making it difficult to provide exact guidance throughout the method (Fig. 2). Therefore, the following suggestions should be adapted to the future study site. We recommend that all *E. coli* isolates obtained from 1-liter spring water samples be tested for antibiotic resistance. Until a strain with at least four antibiotic resistances is found, additional samples should be taken. The initial screening during our study yielded *E. coli* isolates that were multiresistant (i.e., resistant to five antibiotics). The two springs investigated in the present study are located in the same study area and have different contaminant sources (3). Using the same selective media, numerous *E. coli* isolates with resistance against four or more antibiotics were selected, and it was possible to indicate fecal input sites from both springs. Therefore, we conclude that the selective media can be prepared based on only one resistance profile.

The persistence of multiresistant *E. coli* in the environment is different from one multiresistant strain to another. This depends on various factors such as prevalence in the host population, stability of antibiotic-resistant markers, and the selection pressure which occurs at the origin of contamination and in the environment. Although the selection pressure of antibiotic-resistant markers is low in the environment, there are several factors that can cause persistence of markers. Since it is difficult to define how long a strain persists in the environment, we recommend that monitoring be started immediately after the isolation of a suitable strain which is to be used when preparing the selective media (phase I, Fig. 2). We recommend taking samples from the spring and suspected fecal input sites on the same day and repeating the sampling on at least 5 different days (phase II, Fig. 2). The time point of sampling is also important and should be matched to the time point when contamination is most likely to occur. In the present study, we isolated the same multiresistant strain from the LQ spring after rainfall [IV(D), Fig. 4], which was also detected in surface water.

In a previous study, antibiogram and PFGE analyses were applied to compare bacterial isolates from surface water and avian feces (9). Although in that study isolates from both sources were directly analyzed, in our approach selective agar plates with various antibiotics were used to screen for multiresistant *E. coli* prior to further comparative analysis. In this way, strains without any resistance or strains with resistance against one to three antibiotics included only in the selective

media were not isolated. The advantage of this procedure is that the number of isolates was greatly reduced (by at least 90%), and larger sample volumes could be screened for particular multiresistant strains. This makes our method very sensitive, since it is based on a filtration technique that allows for analysis of large volumes of water for particular *E. coli* strains.

Resistance to antibiotics is globally widespread in *E. coli*, and an increase in its prevalence has been observed in recent years (12). It was therefore possible to select two particular multiresistant *E. coli* strains isolated from the LQ spring and generate selective plates based on their resistance profiles. Initially, one selective agar plate (TSA4) containing four antibiotic additives was used for the screening of water samples. Using this plate, the number of strains isolated for further typing was reduced by at least 90% compared to the initial *E. coli* concentration. However, a large number of isolates with variability in their resistance profiles still remained with this approach. Since typing using antibiograms and especially PFGE are time-consuming steps, a second selective plate containing three additional antibiotic additives (TSA7) was prepared based on a strain isolated toward the end of the study. The comparison of the two selective agar plates TSA4 and TSA7 revealed that plates with a higher selectivity (e.g., more antibiotics included) resulted in significantly fewer numbers of isolated strains on one hand and in less variability in the resistance profiles and PFGE profiles among those isolates on the other. In further studies, the preparation of plates for the selection of multiresistant strains should be based on one strain isolated from the spring in question during the preliminary phase of the approach. We conclude that the generated plates must be as selective as possible, with a minimum of four antibiotic additives. However, it has to be considered that the approach may fail to identify fecal inputs from non-point sources, such as wildlife, that may not necessarily harbor antibiotic-resistant *E. coli* populations.

The results obtained by molecular fingerprinting with PFGE confirm that testing for antibiotic resistance alone was not accurate enough to detect particular multiresistant *E. coli* clones. Isolates with the same resistance profiles displayed differences when further typed with PFGE. However, the temporal persistence of PFGE patterns is limited in time. A study by Lu et al. (22) showed that the PFGE patterns of a particular strain changed after 8 weeks, while the patterns of other strains remained stable. Our results revealed the persistence of particular genotypes over a certain period of time, which confirms the suitability of the method over a given time frame. On the other hand, it also shows the discriminatory power of this molecular fingerprinting technique. Isolates that differ by a single genetic event show two to three band differences (13). However, the reliance upon such a difference in PFGE patterns may not be appropriate for a study of this kind. Therefore, only identical PFGE profiles were taken into consideration. Other genotyping methods might provide comparable results to PFGE analysis for determining genetic relatedness among isolates. However, PFGE was selected since it is a well-known and standardized method. The striking discriminatory power and reproducibility of PFGE has made it a widely acceptable method in epidemiology (13). Our approach can be used to link spring water fecal contamination to the source of bacterial input sites. The combination of three criteria—

screening of multiresistant strains and typing using both antibiograms and PFGE—makes this approach sensitive and reliable.

Although hydrogeological tracer tests indicate hydrogeological connections from one point to another, our new approach can be used on a broader scale for the examination of incidents of fecal contamination in drinking water. Since the approach does not indicate direct hydrologic connectivity, a combination with widely used chemical tracers may be useful (5, 17). It is important to keep in mind the complexity of particular water systems, especially in areas with karst geology. Therefore, other criteria, such as geological characteristics and the land use of study areas, should be considered. By combining our approach with hydrogeological measurements, incidents in springs can be extensively investigated.

Detection of fecal input sites. The screening of multiresistant strains and further PFGE-based molecular typing showed that infiltration of the KQ spring by the stream is very likely, and there was an indication that there might be an additional input site from the stream or another location with a hydrologic connection to the stream and the LQ spring.

A particular multiresistant *E. coli* strain found in the KQ spring and at sampling sites upstream and downstream from the wastewater treatment plant showed a possible hydrologic connectivity between surface water and the spring. This finding confirmed prior chemical tracer experiments (3).

The present study provides indications of an additional infiltration of the LQ spring. The same multiresistant *E. coli* strain that was present over 4 days in the LQ spring could be detected in the stream next to the spring, as well as further upstream. Such an interaction has been previously investigated in an extensive study (3). In view of the local geological formation, an infiltration of the Lützel stream into the LQ spring, which was initially considered possible, was not detected. Based on these previously measured hydrogeological parameters (turbidity, conductivity, and discharge) and tracer tests, a connection between the stream and the LQ spring was considered unlikely (3). The multiresistant strain detected in the LQ spring was detected downstream from the WTP but not upstream and could, therefore, conceivably originate at the plant. Since this strain was detected after a rainfall event, weather conditions can be assumed to play an important role in the interaction of the stream and the LQ spring. Previously conducted hydrogeological investigations were performed during low flow conditions (3). However, in order to further investigate possible connections between the stream and the spring, tracer tests after a rainfall event are needed. Our results indicate that either there is an input site from the stream or there is another location with a hydrologic connection with both the stream and the LQ spring. Because the second connectivity was not consistent with previous studies (3), we conclude that further tracer experiments should be performed after a rainfall event in order to confirm our findings and, if necessary, other possible input sites should be considered. Contamination could also have originated from an unknown fecal input site with a subterranean connection to both the spring and stream water sampling sites.

Our results indicate that spring water contamination in the study area originates to a certain extent from surface water. Although the multiresistant strain detected in the KQ spring

did not originate from the wastewater treatment plant, human pathogens could also infiltrate the springs if surface water infiltrates downstream from the wastewater treatment plant. The water supply of Röschenz is mainly derived from the two springs. The impact on human health as a result of spring water contamination can be considered low, since the water is treated in a six-step procedure (flocculation, sedimentation, rapid sand filtration, ozonation, charcoal filtration, and chemical disinfection), but it is of major concern if the treatment process fails. The results provide evidence that the treatment procedure in the study area is essential and should be carefully monitored in order to prevent potential transmission of water-borne diseases from these waters in the future.

Conclusion. The new approach applied was based on the screening of multiresistant *E. coli* in water samples. The selective antibiotic plates used in a first step of the approach reduced time, cost, and effort. Identical isolates could be identified without the construction of large libraries. It was possible to identify identical genotype both in spring and surface water and to confirm previous evidence of infiltration. We therefore conclude that the new approach is helpful in localizing possible input sites for contaminants.

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