

Antibiotic resistance analysis as a means of detecting the origin of faecal pollution in water¹

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Introduction Distribution and availability, the conflict for the use and the quality of water are crucial points, whose importance is still increasing. Problems often arise from the need for supplying clear, clean, drinking water and agricultural practices which can endanger the water quality, either on a chemical or on a microbiological level. In mountain watersheds, the problem is often linked to the presence of grazing herds, or to manuring practices. But other possible sources of microbiological pollution can be the widespread presence of wild animals, especially in protected natural areas, parks and reserves, as well as the presence of man himself, especially in main tourist areas and along most used mountain paths. In Valle d'Aosta, cattle rearing, important wildlife incidence and a widespread presence of tourists coexist. The possibility of pinpointing the different possible sources of faecal pollution would be of important value, either for the attribution of responsibility, or for the prevention or the restoration of water quality. An interesting approach for the classification of possible pollution sources, deriving from the medical practice, is based on the comparison of the antibiotic resistance of faecal bacteria (McKeon *et al.*, 1995; Whitlock *et al.*, 2002; Reinthaler *et al.*, 2003) and, particularly, of faecal streptococci (Wiggins, 1996; Hagedorn *et al.* 1999; Wiggins *et al.*, 1999). Since the patterns of antibiotic resistance may change, following the differences in medical or veterinary treatments which can occur in different areas, and can also vary over time (Wiggins, 1996), the method needs to be adapted to local situations. Within an Interreg project, associating the Administration of the Autonomous Region Valle d'Aosta and the Institut Agricole Régional of Aosta (I), in cooperation with the Service d'Utilité Agricole à Compétence Inter-départementale (SUACI) – Montagne-Alpes du Nord (F), aimed at studying the microbiological quality of natural waters, research was set up to evaluate the feasibility and the reliability of an approach based on the discriminant analysis of multiple antibiotic resistance of faecal streptococci, in Valle d'Aosta.

Materials and methods From June 2004 to April 2005, in Valle d'Aosta, 24 samples of mixed wastes were collected: 12 from cattle, from several cattlesheds, both from dairy and beef cattle, 6 from wildlife (chamois, ibex, deer, fox) and 6 from human wastes, from the sewage arriving to the Aosta wastewater treatment plant. This facility processes only human waste, but the possibility of agricultural input from surface runoff cannot be excluded.

The strains of faecal streptococci were isolated from these samples. Adapting the methodology proposed by Wiggins (1996), the isolates were submitted to each of 4 concentrations of 8 antibiotics, whose widespread use in veterinary and human medicine was also verified with the aid of the medical and veterinary services of Aosta. The following concentrations were used: 5, 10, 15 and 30 µg/ml for amoxicillin (AMO) and vancomycin (VAN); 5, 10, 30 and 50 µg/ml for lincomycin (LIN) and spiramycin (SPI); 1, 10, 20 and 50 µg/ml for cefaclor (CEF); 1, 5, 10 and 20 µg/ml for ofloxacin (OFL); 0.1, 1, 10 and 20 µg/ml for penicillin G (PEN); and 10, 15, 30 and 50 µg/ml for tetracycline (TET).

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Data on the growth of each isolate in the presence of each concentration of each antibiotic (32 variables per isolate) were treated with the discriminant analysis of the SPSS statistical programme. The classification table showed the numbers and percentages of correctly classified items, after cross-validation, as well as those of misclassified isolates for a given source (false negatives) and those of isolates from other sources that were misclassified as a given source (false positives).

Results From the 24 samples, a total of 3,256 isolates were obtained and their patterns of antibiotic resistance were determined and analysed (Table 1).

Table 1. Patterns of antibiotic resistance of faecal streptococci isolated from various sources

Drug and concentration ($\mu\text{g/ml}$)	% of resistant isolates from each source		
	Cattle ($n=1,208$)	Wild ($n=992$)	Human ($n=1,056$)
Amoxicillin			
5	9	0	0
10	2	0	0
15	0	0	0
30	0	0	0
Cefaclor			
1	70	97	96
10	61	68	94
20	47	57	93
50	32	48	36
Lincomycin			
5	60	48	55
10	45	30	41
30	13	3	4
50	5	0	0
Ofloxacin			
1	61	74	75
5	6	20	11
10	1	9	1
20	0	1	0
Penicillin G			
0.1	83	98	96
1	58	96	94
10	15	31	33
20	11	17	18
Spiramycin			
5	5	15	2
10	4	3	2
30	4	0	1
50	4	0	1
Tetracycline			
10	20	10	2
15	20	7	2
30	19	7	2
50	17	4	1
Vancomycin			
5	11	5	0
10	2	5	0
15	0	5	0
30	0	0	0

A relatively high resistance to PEN, CEF and, to a lesser extent, to OFL was recorded for isolates of all 3 sources but especially for wild and human ones. On the other hand, these isolates were very sensitive to AMO, even at the lowest concentrations. Human isolates were also the least resistant to SPI, TET and VAN and cattle ones were generally the most resistant to LIN and TET. The overall resistance levels are lower than the values reported in bibliography (Wiggins, 1996; Hagedorn *et al.* 1999): that could be explained by a relatively moderate utilisation of antibiotics, particularly in veterinary medicine, in this region.

In wild samples, the relatively high resistance was mainly due to isolates from faeces of foxes and ibexes living close to human settlements.

The discriminant analysis correctly classified 85.5%, 82.2% and 72.5%, respectively, cattle, human and wild isolates (Tab. 2).

Table 2. Cross-validated discriminant analysis of antibiotic resistance profiles of faecal streptococci isolated from various sources

Source	no. of isolates	No. (%) of isolates classified as:					
		Cattle		Wild		Human	
		no.	%	no.	%	no.	%
Cattle	1,208	1,033	85.5	102	8.4	73	6.0
Wild	992	79	8.0	719	72.5	194	19.6
Human	1,056	72	6.8	116	11.0	868	82.2

Cattle isolates were quite accurately classified, while a certain confusion was observed regarding wild and human isolates: more than 10% of human isolates were classified as wild and the opposite occurred for nearly 20% of wild ones. The overall average rate of correct classification (ARCC), calculated as the ratio of correctly classified data to the total number of isolates, was 80.5%. If all the isolates were grouped in only 2 categories (i.e. cattle vs. wild+human), the ARCC improved to 89.8% (Tab 3). This pooling may be useful when it is necessary to determine if the source of the pollution is an agricultural one (grazing animals, manuring) or not.

Table 3. Cross-validated discriminant analysis of antibiotic resistance profiles of faecal streptococci isolated from cattle and non-cattle sources

Source	no. of isolates	No. (%) of isolates classified as:			
		Cattle		Wild+Human	
		no.	%	no.	%
Cattle	1,208	1,042	86.3	166	13.7
Wild+Human	2,048	166	8.1	1,882	91.9

Conclusions The discriminant analysis of multiple antibiotic resistance confirmed its effectiveness in distinguishing the sources of faecal streptococci and could be a useful tool for pinpointing, to a suitable level of precision, the source of unknown faecal pollutions in natural waters in Valle d'Aosta. Nevertheless, a larger sampling is required to better characterize the resistance of faecal streptococci associated to wildlife.

The reduction of the number of drugs utilised and/or of the concentrations tested could be profitable for the simplification and the speeding up of the methodology, and will be the focus of future investigations.

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Summary The microbiological quality of mountain water is occasionally endangered by faecal bacteria. In mountain watersheds, nonpoint sources of microbiological pollution can be linked to the presence of domestic herbivores, of wild animals or of man himself. A study was conducted to explore the reliability of antibiotic resistance analysis as a method to determine the origin of faecal pollution, discriminating among the 3 different sources. A set of 3,256 faecal streptococci isolates from known livestock (12 samples, 1,208 isolates), wildlife (6 samples, 992 isolates) or human sources (6 samples, 1,056 isolates) was submitted to 4 concentrations of 8 antibiotics (amoxicillin, cefaclor, lincomycin, ofloxacin, penicillin G, spiramycin, tetracycline, vancomycin) and their ability to grow was measured. The cross-validated discriminant analysis of the resistance patterns correctly classified 80.5% of the known isolates into one of the 3 possible sources. In particular, 85.5% of cattle, 72.5% of wild and 82.2% of human isolates were correctly classified. When the isolates were pooled into 2 possible categories (cattle vs. wild+human), the average rate of correct classification increased to 89.8%: 86.3% of cattle and 91.9% of wild+human isolates were correctly classified. The use of discriminant analysis of antibiotic resistance patterns is confirmed as being a promising method to determine the sources of faecal pollutions in water.

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