Survey on antimicrobial residues in raw milk and antimicrobial use in dairy farms in the Emilia-Romagna region, Italy

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Abstract

This survey investigated the antimicrobials most commonly used in dairy herds and antimicrobial residues most frequently detected in milk to evaluate the suitability of rapid screening tests to determine antimicrobial residues in milk. The investigation was carried out in 45 dairy herds consulting the farm administration records and in a national dairy industry collecting milk from almost all the dairy farms studied. Data were recorded on: i) treatments with drugs containing antimicrobials during the 12 months prior to the visit; ii) antimicrobial active substances present in the drugs; iii) data from routine controls to detect antimicrobial residues (52,771 samples). The antimicrobial classes most commonly used were penicillins, cephalosporins, fluoroquinolones, macrolides, sulphonamides, tetracyclines, aminoglycosides and lincosamides; the most frequently used antimicrobial not belonging to any of the previous groups was rifampicin. Sixty-four samples collected from milk trucks yielded antimicrobial residues exceeding the detection limit of the screening test used: sulphonamide residues were the most prevalent (3.4%), followed by tetracycline (0.3%) and penicillins and cephalosporins (0.03%). The antimicrobial classes most commonly used on dairy farms are the same as the residues most frequently detected in milk. The association of several commercially available rapid test kits proved satisfactory for determination of the veterinary antimicrobial drugs most used on dairy farms but at least five kits are required.

Therefore, knowledge of the most frequently used veterinary drugs and periodic monitoring are required for the dairy industry to develop a targeted and effective control plan.

Introduction

Antimicrobial agents are widely used in dairy cattle management for prophylactic and therapeutic purposes. Although the use of these drugs has significantly improved the health and production efficiency of food-producing animals (Alomirah et al., 2007), incorrect use, improper administration or mistakes by farmers and veterinarians in observing the withdrawal time for treated animals can result in antimicrobial residues in milk and dairy products. Antimicrobial residues are currently the most common inhibitory substances detected in milk, and may have undesirable effects on milk quality, milk technological properties, dairy product quality, and last but not least human health (Ortelli et al., 2009, Piñero et al., 2012, Zhan et al., 2012).

The importance of monitoring antimicrobial residues in milk is emphasized by the role of milk and dairy products in human nutrition (Ortelli et al., 2009). The European Union (EU) has established safe maximum residue limits (MRLs) for pharmacologically active substances in foodstuffs of animal origin (European Commission, 2010). Several chromatographic confirmatory methods, which must comply with Commission Decision 657/2002/EC, have been described for the determination of antimicrobials in food. However, all routine monitoring tests on raw milk in bulk tank trucks should be completed as soon as possible (usually in less than 3 h) before the milk is unloaded for processing. Monitoring a large number of milk samples for the presence of residues requires rapid low cost screening methods. Dairy plant monitoring is primarily performed using microbiological and immunological screening methods (Bilandžić et al., 2011; Zhan et al., 2012) but these techniques present some drawbacks. Microbiological screening methods may have an analytical sensitivity lower than the MRLs allowed by law, while immunological screening methods require one test kit for each family of antimicrobials and the sensitivity depends on the cross-reactivity of each compound resulting in very different detection limits within the same drug family (Ortelli et al., 2009).

The European Regulation No. 178/2002/EU (European Commission, 2002) states that in EU the food business operators have the primary legal responsibility for ensuring food safety, and it is generally recognised that producers are in the best position to devise methods for warranting the food safety (Vragović et al., 2011). In dairy plants, it may be difficult to implement a control programme to avoid residues of veterinary antimicrobials in milk due to the large number of different compounds and formulations commercially available. Knowledge of the antimicrobial substances most frequently used in dairy farms will serve to choose appropriate screening tests to detect antimicrobial residues in milk. The aim of the survey was therefore: i) to monitor the antimicrobial agents most frequently used in dairy farms in the Emilia-Romagna region and to evaluate the ability of the screening kits used for veterinary drug residue determination in raw milk to detect residues of these antimicrobial agent; ii) to evaluate the agreement of the data collected on the most frequently used antimicrobials with the data on the most common antimicrobial residues tested in milk by a national dairy industry; iii) to evaluate the availability of screening test kits able to detect potential residues of the most frequently used antimicrobial drugs.

Materials and methods

The survey was conducted in 45 dairy farms located in the Emilia-Romagna region in northern Italy and data were collected between January and December 2012. Milk was primarily produced for the dairy industry (destined for pas-
Antimicrobial residues in raw milk

Our study showed that the dairy farms investigated used a total of 43 veterinary drugs with 32 active substances recorded in the commercially available formulations. Table 2 summarizes the list of active substances and their frequency of use in the dairy farms. The total frequency of these active substances was 16,972 (Total=n number of treated animals' treatment days); 16 active substances showed F>250 and on the whole a F equal to 15,331, i.e., 90% of the F total. The remaining 16 active substances not included in the main active substances were marbofloxacin, dihydrostreptomycin, neomycin, cephalexin, sulphamerazine, cephapirin, spiramycin, trimethoprim, sulphamethopyrazin, thiamphenicol, cepheperazone, penethamate, benetaminpenicillin, framyctin, sulphahippuride, and naphcillin.

The antimicrobial agents used belonged to the following classes: lactams (51.3% of the total use of antimicrobial agents), fluoroquinolones (8.8%), macrolides (4.6%), sulfonamides (3.1%), tetracyclines (2.6%), lycosamides (2.1%) and amphenicals (0.3%); other antimicrobial agents used not belonging to the previous reported classes were rifampicin, clavulanic acid and trimethoprim (respectively 12.4, 11.2 and 0.5% of the total antimicrobial agents used).

### Results

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### Table 1. Number of milk samples analysed and sensitivity of different kits for the antimicrobial substances tested during routine controls performed by a national dairy industry in 2012 and maximum residue limits regulated by European Regulation 37/2010/EU.

| Active Substance | Tests used | Pharmacologically active substance | Sensitivity charm,° | Sensitivity DelvotestSP°, µg/kg | MRL, µg/kg |
|------------------|------------|-----------------------------------|---------------------|---------------------------------|------------|
| **β lactams**    |            |                                    |                     |                                 |            |
| 45,677           | Charm MRL/ Delvotest SP | Anoxicillin            | 3 - 4               | 3-5                             | 4          |
|                  |            | Ampicillin                    | 3 - 4               | 3-5                             | 4          |
|                  |            | Cephalacetril               | 8 - 18              | 20-40                           | 125        |
|                  |            | Cephalexin                  | 30 - 60             | 60-100                          | 100        |
|                  |            | Cephalonium                  | 3 - 5               | 10-25                           | 20         |
|                  |            | Cephabolin                  | 12 - 20             | no claim                        | 50         |
|                  |            | Cephoperazone               | 5 - 9               | 60-100                          | 50         |
|                  |            | Cepfoquinome                | 15 - 20             | no claim                        | 20         |
|                  |            | Ceflurofur                  | 10 - 20             | 50-70                           | 100        |
|                  |            | Cephuroxime                 | 3 - 5               | no claim                        | nr         |
|                  |            | Cephapirin                  | 6 - 10              | 5-10                            | 60         |
|                  |            | Cloxacillin                 | 25 - 35             | 15-25                           | 30         |
|                  |            | Dicloxacillin               | 20 - 30             | 10-15                           | 30         |
|                  |            | Penicillin G                | 2 - 3               | 2.5                             | 4          |
|                  |            | Oxacillin                   | nr                  | 10                              | 30         |
|                  |            | Naphcillin                  | nr                  | 5-10                            | 30         |
| **Enrofloxacin** | 759        | Charm Enrofloxacin Test      | Enrofloxacin        | 8                               | 100        |
| **Tetracycline** | 3752       | Charm Tetracycline Test      | Chlortetracycline   | 50-100                          | 100        |
|                  |            | Oxytetracycline             | 50-100              | 100                             |            |
|                  |            | Tetracycline                | 10-30               | 100                             |            |
| **Sulphonamides**| 1040       | Charm Sulfa Test            | All substances      | 100                             | 100        |
| **Streptomycin** | 765        | Charm Streptomycin Test      | Streptomycin        | 75                              | 200        |
|                  |            | Dihydrostreptomycin         | 75                  | 200                             |            |

°Detection limit reported in literature (Bilandžić et al., 2011) or by manufacturers (Charm, 2013; DSM, 2013; Tecuala, 2013; Zeulab, 2013; Neogen, 2013; Globalcube, 2013); MRL, maximum residue limit; nr, not reported.
A total of 64 samples yielded antimicrobial residues exceeding the detection limit of the screening test used (Table 3). Briefly, sulphonamide residues were most prevalent, present in 36 samples (3.4%), followed by tetracycline in 13 samples (0.3%) and β-lactams detected in 15 samples (0.03%). The analysis of commercially available rapid test kits showed that they were able to detect 14 out of 16 antimicrobial substances most frequently used (F>250) in the Emilia-Romagna region, except for clavulanic acid and rifampicin. To determine the antimicrobial agents less used on the dairy farms studied, test kits are commercially available for the determination of 13 out of 16 antimicrobial substances, except for sulphonamide, benetaminpenicillin and framycetin.

### Discussion

Our data on the frequency of antimicrobial use in the dairy farms surveyed partly overlap data reported by the Official Veterinary Service during a monitoring performed in the Emilia-Romagna region (Moro et al., 2006), in which β-lactams, macrolides, aminoglycosides, polymyxins, sulphonamides, amphenicols and tetracyclines were the most used antimicrobial classes in bovine herds. Our study found that penicillins, cephapenem, fluoroquinolones, macrolides and sulphonamides respectively were the most used classes of antimicrobials. The discrepancies in the two studies depend on their different aims and the consequent different data collection methodology on antimicrobial use: in the study of Moro et al. (2006) data on antimicrobial use in both beef and dairy production were collected, instead, in our study only dairy farms were taken into account in order to record the veterinary drugs that could potentially result in antimicrobial residues in milk and milk products. As the most common diseases differ in beef and dairy production, disease management (antimicrobial substances, dosage, associations, route of administration, etc.) may also differ. The use of antimicrobials in dairy farms varies in different countries (Hill et al., 2009; González et al., 2010; Saini et al., 2012), and this may depend on the different Regulations in force. Our findings can be roughly compared to the study by Hill et al. (2009) in the USA which reported that the most common primary treatments for both lameness and respiratory disease in lactating cows were penicillins, cephapenem and tetracyclines, whereas cephapenem were the most common primary treatment for mastitis, closely followed by penicillins and then macrolides. González et al. (2010) reported that penicillins, sulphonamides, aminoglycosides and tetracyclines were the most frequently used active substances in Switzerland, whereas use of third and fourth generation cephapenem, fluoroquinolones and macrolides was low.

Cephapenem, penicillin combinations, and tetracyclines were the most commonly used antimicrobial drug classes on Canadian dairy farms (Saini et al., 2012) similar to findings on Dutch dairy farms (Mevius et al., 2009). Generally speaking, β-lactams were used on all dairy farms and constituted the highest proportion of antimicrobials with cephapenem used at a higher rate than penicillins. The comparison of data on the antimicrobials most commonly used in dairy herds with the data collected on the antimicrobial residues most frequently detected in milk in this study showed a good overlap: few data have been reported by the rapid alert system for food and feed (RASFF) on veterinary drug residues in milk and milk products in recent years. Literature reports are also scarce: Bilandžić et al. (2011) monitored Croatian veterinary drug residues from 2008 to 2010, reporting a low frequency of positive samples (<1%) belonging to sulphonamides, tetracycline, aminoglycosides and fluoroquinolones. Considering no β-lactam or rifampicin residue determination was performed in the Croatian study, there may be a non-negligible overlap with our data both in veterinary antimicrobial drugs used in dairy farms and residues in milk in which sulphonamides, tetracycline, and β-lactams

### Table 2. List of active substances and frequency of use in the dairy farms studied.

| Antimicrobial agent | Usage frequency | % | Associations with other antibiotics (number) |
|---------------------|-----------------|---|---------------------------------------------|
| Amoxicillin         | 2985            | 17.59 | Clavulanic acid (2) |
| Rifampicin          | 2109            | 12.43 | Cepacetril (1) |
| Clavulanic acid     | 1908            | 11.24 | Anoxicillin (2) |
| Ampicillin          | 1296            | 7.64  | |
| Enrofloxacin        | 1281            | 7.55  | |
| Dicloxacillin       | 963             | 5.67  | Ampicillin (2) |
| Cephacetrile        | 855             | 5.04  | Rifampicin (1) |
| Ceftiofur           | 849             | 5.00  | |
| Cephquinome         | 639             | 3.77  | |
| Oxytetracycline     | 453             | 2.67  | |
| Cloxacillin         | 423             | 2.49  | |
| Lyncomycin          | 372             | 2.19  | Spectinomycin (1) |
| Spectinomycin       | 372             | 2.19  | Lyncomycin (1) |
| Tylosin             | 312             | 1.84  | |
| Sulphonamethazine   | 261             | 1.54  | Sulphamerazine (1); Trimethoprim |
| Penicillin G        | 253             | 1.49  | Dihydro-streptomycin (3); Naphcilin (1) |
| Others              | 1641            | 9.67  | |
| Total               | 16,972          | 100   | |

*Number of animals treated with the antimicrobial agent x total treatment days for each treatment in the period of observation (1 year); number of veterinary drugs in which the antimicrobial agents are associated in commercial formulation (data regarding our observations).*

### Table 3. Number of tested antimicrobial residues exceeding the maximum residue limits permitted in raw milk samples destined for pasteurization collected in the Emilia-Romagna region in 2012.

| Antibiotic detected with rapid method | Number of positive samples | Percentage of positive samples |
|--------------------------------------|-----------------------------|-------------------------------|
| β-lactams                            | 15                          | 0.03                          |
| Enrofloxacin                         | 0                           | 0.00                          |
| Tetracycline                         | 13                          | 0.34                          |
| Sulphonamides                        | 36                          | 3.46                          |
| Streptomycin                         | 0                           | 0.00                          |
| Total                                | 64                          | 0.12                          |
were detected. However, aminoglycosides were not investigated in the Croatian survey.

The antimicrobial residues in pasteurized milk from Brazil observed by Zanella et al. (2010) included aminoglycosides, tetracycline and β lactams, and were similar to our findings, except for the high antimicrobial residues (30.8%) which were, however, below the MRLs permitted in Brazil.

Nowadays, rapid test kits (microbiological or immunoreceptor-based) for the common antimicrobial drug residues in milk are increasingly used and the risk of milk contamination not detected by an available screening test is very limited. No validated test kits are available for several antimicrobial substances but the commercial formulation must be taken in account for evaluating the risk of the presence of residues: clavulanic acid is sold exclusively in milk and dairy products in the state of Kuwait. J. Food Quality 30:745-763.

Bilandžić, N., Kolanović, R.S., Varenina, I., Scortichini, G., Annunziata, L., Brestilo, M., Rudan, N., 2011. Veterinary drug residues determination in raw milk in Croatia. Food Control 22:1941-1948.

Charm, 2013. Available from: http://www.charm.com/en/dairy/dairy-antibiotics.html. Accessed January 2013.

European Commission, 2002. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, 657/2002/EC. In: Official Journal, L 221, 17/08/2002, pp 8-36.

European Commission, 2010. Commission Regulation of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, 37/2010/EU. In: Official Journal, L 15, 20/01/2010, pp 1-72.

Globalcube, 2013. Available from: https://www.globalcube.net/clients/beldico/content/medias/products/s_imp/lab/diagnostics/dairy/copan_copan_milk_test.pdf.

González, S.M., Steiner, A., Gassner, B., Regula, G., 2010. Antimicrobial use in Swiss dairy farms: quantification and evaluation of data quality. Prev. Vet. Med. 95:50-63.

Hill, A.E., Green, A.L., Wagner, B.A., Dargatz, D.A., 2009. Relationship between herd size and annual prevalence of and primary antimicrobial treatments for common diseases on dairy operations in the United States. Prev. Vet. Med. 88:264-277.

Mevius, D.J., Koene, M.G.J., Wit, B., van Pelt, W., Bondt, N., 2009. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2008. Available from: http://www.wageningenur.nl/en/Publication-details.htm?publicationId=publication-way-333935393035

Moro, M.L., Palazzi, M., Buttazzi, R., Diegoli, G., 2006. Use of antimicrobics and other veterinary drugs in livestock in Italy. J. Vet. Med. A 53:772-783.

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Conclusions

The association of several commercially available rapid test kits proved satisfactory for determination of the most used veterinary antimicrobials but at least three kits are required. For the determination of antimicrobial substances less frequently used further two test kits are needed. An effective control plan to avoid the presence of veterinary drug residues requires frequent milk monitoring and the use of several test kits making antimicrobial testing expensive and time-consuming. Our results show that the antimicrobial classes most commonly used in dairy farms are the same as those most frequently detected as residues in milk. Therefore knowledge of the most frequently used veterinary drugs and periodic monitoring are required for the dairy industry to develop a targeted and effective control plan to avoid processing milk containing veterinary drug residues. No currently validated screening test kits are commercially available for the five most common antimicrobial substances.

References

Alomirah, H., Al-Mazeedi, H., Al-Zenki, S., Al-Aati, T., Al-Otaibi, J., Al-Batel, M., Sidhu, J., 2007. Prevalence of antimicrobial residues in milk and dairy products in the state of Kuwait. J. Food Quality 30:745-763.

Bilandžić, N., Kolanović, R.S., Varenina, I., Scortichini, G., Annunziata, L., Brestilo, M., Rudan, N., 2011. Veterinary drug residues determination in raw milk in Croatia. Food Control 22:1941-1948.

Charm, 2013. Available from: http://www.charm.com/en/dairy/dairy-antibiotics.html. Accessed January 2013.

DSM, 2013. Available from: http://www.fortrichard.co.nz/editor/assets/Downloads/developvote%20technical%20bulletin.pdf. Accessed January 2013.

European Commission, 2002. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, 657/2002/EC. In: Official Journal, L 221, 17/08/2002, pp 8-36.

European Commission, 2010. Commission Regulation of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, 37/2010/EU. In: Official Journal, L 15, 20/01/2010, pp 1-72.

Globalcube, 2013. Available from: https://www.globalcube.net/clients/beldico/content/medias/products/s_imp/lab/diagnostics/dairy/copan_copan_milk_test.pdf.

González, S.M., Steiner, A., Gassner, B., Regula, G., 2010. Antimicrobial use in Swiss dairy farms: quantification and evaluation of data quality. Prev. Vet. Med. 95:50-63.

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