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Resistance profiles and genetic diversity of *Escherichia coli* strains isolated from acute bovine mastitis

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Summary

Between March 2011 and February 2012 83 *E. coli* strains were isolated from mastitis milk samples from 83 different animals (67 farms) and tested for their sensitivity to various antibiotics by means of disk diffusion method and genotyped by determination of the phylogenetic groups as well as by pulsed field gel electrophoresis (PFGE). The antibiotics were chosen on the basis of their licenses for intramammary application in Switzerland. As many as 16.9 % of the isolates were resistant to one or more antimicrobial agents. Amoxicillin-clavulanic acid, gentamicin and third generation cephalosporins proved effective against the majority of these strains. Nevertheless, one *bla*$_{\text{CTX-M-14}}$ harbouring extended-spectrum-beta-lactamase producing strain was found. Genetic analysis grouped most of the strains (87 %) into phylogenetic groups A and B1. PFGE genotyping demonstrated that *E. coli* from cows with mastitis even from the same farm were genotypically very diverse.

Keywords: *E. coli*, mastitis, resistance, genotyping, phylogenetic group, PFGE

Resistenzprofile und genetische Vielfalt von *Escherichia coli* Stämmen isolierst aus akuten bovinen Mastitiden

Zwischen März 2011 und Februar 2012 wurden 83 *E. coli* Stämme von 83 verschiedenen Kühen aus 67 Betrieben gesammelt und auf ihre Empfindlichkeit gegenüber verschiedenen Antibiotika getestet. Die Antibiotika wurden aufgrund der Zulassung für eine intramammäre Applikation in der Schweiz ausgesucht und die Empfindlichkeitstestung mittels Agardiffusions-Methode durchgeführt. Zudem wurden alle Stämme hinsichtlich ihrer Zugehörigkeit zu den phylogenetischen Gruppen wie auch mittels Pulsfeldgelelektrophorese (PFGE) genotypisiert. 16.9 % aller Stämme zeigten Resistenzen gegenüber einem oder mehreren Antibiotika. Amoxicillin-Clavulansäure, Gentamicin und Cephalosporine der dritten Generation erwiesen sind als wirksam gegen die Mehrheit der *E. coli* Stämme. Jedoch wurde ein extended-Spectrum-beta-Lactamase Bildner, welcher das *bla*$_{\text{CTX-M-14}}$-Gen trägt, gefunden. Die genetische Analyse gruppierte das Gros der Stämme (87 %) in die phylogentischen Guppen A und B1. Die weitere Genotypisierung mittels PFGE zeigte eine grosse Diversität unter den *E. coli* Stämmen, auch wenn diese vom selben Betrieb stammten.

Schlüsselwörter: *E. coli*, Mastitis, Resistenz, Genotypisierung, phylogenetische Gruppen, PFGE

Introduction

Mastitis remains a major challenge to the worldwide dairy industry. For Switzerland, the average annual cost due to clinical mastitis has been estimated to be about SF 300.– per cow (Rüsch, 1995). *Escherichia coli* is the most common Gram-negative bacterium causing acute mastitis in cows worldwide (Hogan and Smith, 2003, Ericsson Unnerstad et al., 2009). *E. coli* induced mastitis is characterized as a relatively short-term disease process and induces a distinct acute phase response.

Antimicrobial treatment of an acute clinical mastitis has to start before the results of antimicrobial susceptibility testing are available because of the very often peracute course of the disease. Knowledge on current resistance patterns guides this "empiric" treatment and will enable a more accurate use of antibiotics. The latest published data on resistance profiles of *E. coli* causing mastitis in cows in Switzerland are about 10 years old (Stephan and Rüsch, 1997; Corti et al., 2003). In the meantime extended spectrum β-lactamases (ESBL) producing *E. coli* isolated from milk of cows with clinical mastitis were described

(Locatelli et al., 2009). And recently, a CMY-2 β-lactamase producing *E. coli* (plasmid-mediated AmpC-producing *E. coli*) isolated from a cow with recurrent mastitis, was found in Switzerland (Endimiani et al., 2012). Therefore, from a clinical perspective, current data about resistances profiles of *E. coli* are required.

The *E. coli* species encompasses both pathogenic and non-pathogenic strains. Pathogenic strains cause a variety of enteric and extraintestinal infections in humans and animals, mostly in a host- or organ-specific way. Nevertheless, with regard to bovine mastitis, an *E. coli* pathogenic subset has not been identified yet. Bovine mastitis *E. coli* do not belong to specific antigen O serogroups (Wenz et al., 2006) and are not biochemically different from fecal *E. coli* (Nemeth et al., 1994). Because of this apparent lack of specific features, it has been largely accepted that there is no strain specificity in *E. coli* bovine mastitis and that various *E. coli* strains found in the environment bear the same potential to cause the disease (Burvenich et al., 2003, Fernandes et al. 2011). The aim of this study was to retrieve current data on resistance profiles of *E. coli* strains form bovine mastitis milk samples as well as on the degree of genetic variability among isolates in Switzerland.

Material and Methods

Strains

A total of 83 *E. coli* strains from acute clinical cases of bovine mastitis were isolated from 83 different animals (67 different farms distributed in the cantons of Zurich, Graubünden, Thurgau, Aargau, St. Gallen, Uri and Zug). Not more than two cows per farm were included. The isolates were collected between March 2011 and February 2012. Milk samples were taken during farm calls by the attending veterinarian from the affected quarter of each cow in an aseptical manner.

Using a sterile loop, the samples were streaked onto sheep blood agar base (Beckton Dickinson AG, Allschwil, Switzerland), supplemented with 5% sheep blood (Oxoid, Pratteln, Switzerland) as well as onto BROLACIN-Agar (VWR International AG, Dietikon, Switzerland) and incubated at 37 °C overnight. The *E. coli* strains were confirmed by colony morphology, Gram stain, and biochemical tests such as acid production from mannit, ONPG test, tests for urease, indol and H_2S production and the lysindecarboxylase test. The strains were stored at –80 °C.

Susceptibility Testing

The strains were subjected to antimicrobial susceptibility testing using the standard disk diffusion test according to the protocols recommended by the Clinical and Laboratory Standards Institute (2008). The antimicrobial agents tested as well as the corresponding interpretative criteria are summarized in Table 1. Bacteria were grown 4–5 h in BHI (Brain Heart Infusion, Oxoid, Pratteln, Switzerland) and diluted to 0.5 Mac Farland turbidity by comparison with a standard. Thereafter, bacterial suspensions were swabbed uniformly across a Müller-Hinton-Agar (Oxoid, Pratteln, Switzerland) plate and the filter-paper disks impregnated with the antimicrobial were placed on the surface of the agar using a dispenser. The disks were provided from Beckton Dickinson AG, Allschwil, Switzerland, except Ceftiofur, which was provided from Oxoid, Pratteln, Switzerland. Plates were incubated at 35 °C for 18 h and the zone of inhibition (in mm) was assessed and measured using a calliper. Interpretive criteria according to the performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals (Clinical and Laboratory Standards Institute, 2008) were used. Accuracy of the test system was monitored by including the reference strain *E. coli* ATCC 25922.

Additionally the strains were cultured on Brilliance ESBL agar (Oxoid, Pratteln, Switzerland) and incubated for 24 h at 37 °C. Strains, which were able to produce blue colonies, were confirmed as ESBL producers on Muller-Hinton agar plates using E-Test-ESBL strips containing cefotaxime, cefepime or ceftazidime each alone and in combination with clavulanic acid (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's recommendations.

Table 1: Antimicrobial agents used in this study, interpretative criteria[1] and prevalence of resistances (n = 83).

Antimicrobial agents		resistant (inhibition zone diameters in mm)	susceptible (inhibition zone diameters in mm)	resistant strains (%)
Gentamicin (GM)	10 µg	≤12	≥15	2.4
Kanamycin (K)	30 µg	≤13	≥18	10.8
Ampicillin (AM)	10 µg	≤13	≥17	15.7
Amoxicillin-clavulanic acid (AC)	20/10 µg	≤13	≥18	0
Cephalothin (CF)	30 µg	≤14	≥18	3.6
Ceftiofur (CFT)	30 µg	≤17	≥21	1.2

[1] according to M31-A3: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard-Third Edition; CLSI, Wayne, USA 2008.

Identification and sequencing of *bla*ESBL genes

Bacterial strains confirmed for producing ESBLs were further analysed by PCR and by sequencing the whole open reading frames (ORF) of *bla* genes. DNA was extracted by a standard heat lysis protocol. Thereafter, specific primer sets were used to search for β-lactamase-encoding genes belonging to *bla*TEM, *bla*SHV and *bla*CTX-M families (Geser et al., 2011; Geser et al. 2012). Resulting amplicons were purified using the PCR Purification Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's recommendations. Custom-sequencing was performed by Microsynth (Balgach, Switzerland) and the nucleotide and protein sequences were analyzed with Codon Code Aligner V. 3.7.1.1. For database searches the BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov/blast/) was used.

Determination of the *E. coli* phylogenetic groups

Phylogenetic analyses have shown that *E. coli* strains fall into four main phylogenetic groups (A, B1, B2, D). All isolates in the collection were assigned to phylogenetic groups, in which group A/B1 typically contained commensal isolates and group B2/D isolates were associated with virulence, using a triplex PCR protocol (Clermont et al., 2000) after DNA extraction by a standard heat lysis protocol.

PFGE

Genetic variability of isolates was determined by macrorestricion analysis and pulsed field gel electrophoresis (PFGE) according to the PulseNet standardized protocol for the subtyping of *E. coli* O157, *Salmonella*, *Shigella* (Ribot et al., 2006). After electrophoresis the gels were ethidium bromide stained and the banding pattern was photographed under (UV) illumination and a digital image (that was converted to TIFF format) of the pattern was acquired using the GelDoc system (Bio-Rad). The TIFF images were analysed using the BioNumerics software GelCompare (Applied Maths, Sint-Martens-Latem, Belgium) and Dice coefficient and UPGMA was employed to generate dendrograms. Analysis parameters were set to 2 % for both optimization and tolerance values respectively.

Results and Discussion

Susceptibility Testing

Prevalence data for resistances in view of the antibiotics tested are summarized in Table 1. In total, 16.9 % (n = 14) of the strains showed resistance to one or more antimicrobial agent: 2.4 % (n = 2) of all *E. coli* strains were resistant against gentamicin, 10.8 % (n = 9) were resistant against kanamycin, 15.7 % (n = 13) against ampicillin, 3.6 % (n = 3) against cephalothin and 1.2 % (n = 1) against ceftiofur. No resistant strains were found for the combination amoxicillin-clavulanic acid. Nine strains (10.8 %) were resistant against more than one class of antibiotics, the most frequent ones showed resistance against kanamycin and ampicillin (7.2 %, n = 6). One strain (1.2 %) showed resistance against kanamycin, ampicillin and cephalothin and another strain (1.2 %) showed resistance against gentamicin, kanamycin and ampicillin. In addition we found one ESBL-producer, harbouring *bla*CTX-M-14, which was also resistant against the aminoglycosides kanamycin and gentamicin. The distributions of the inhibition zones of all strains are shown in Figure 1.
A comparison of the results from this study with previous studies in Switzerland (Stephan and Rüsch, 1997, Corti et al., 2003) revealed no global changes in the resistance situation during the last 15 years. However, concerning cephalothin, 22 strains fell into the interpretative category "intermediate". This might indicate a shift of the population towards resistance and will need to be observed over the next years. By comparing the results of this study with recently published data from European countries (Hendriksen et al., 2008; Botrel et al., 2010) no obvious differences in view of resistance prevalence were evident.

Phylogenetic groups

Most of the isolates investigated could be assigned to phylogenetic groups A and B1, with 29/83 (34.9 %) assigned to group A and 43/83 (51.8 %) assigned to group B1. Eleven isolates (13.3 %) were assigned to the group D and none to phylogenetic group B2. With regard to virulence, phylogenetic groups B2 and D are considered to be more likely to carry pathogenicity-associated genes, while groups A and B1 are classified commensal strains (Clermont et al., 2000). In our study, most of the isolates belonged to groups A and B1. In this context, it is worth mentioning, that this was in agreement with findings from a survey showing that *E. coli* isolates, shed by healthy cattle predominantly belonged to lineages A and B1 (Houser et al., 2008). However, it cannot be excluded that such strains may harbour some factors which may favour e.g. colonization of the udder.

Genotyping

The genetic variability was high among the 83 *E. coli* mastitis isolates from the 67 farms, and most of the strains were clonally not related based on a cut off value of 95 % (Fig. 2). Even isolates from multiple cows from the same farm displayed diverse PFGE patterns. Only three PFGE patterns (two strains each were indistinguishable) were found in different farms (Fig. 2). Our data confirm the results of Wenz et al. (2006), who also found a high heterogeneity among *E. coli* strains isolated from cows with

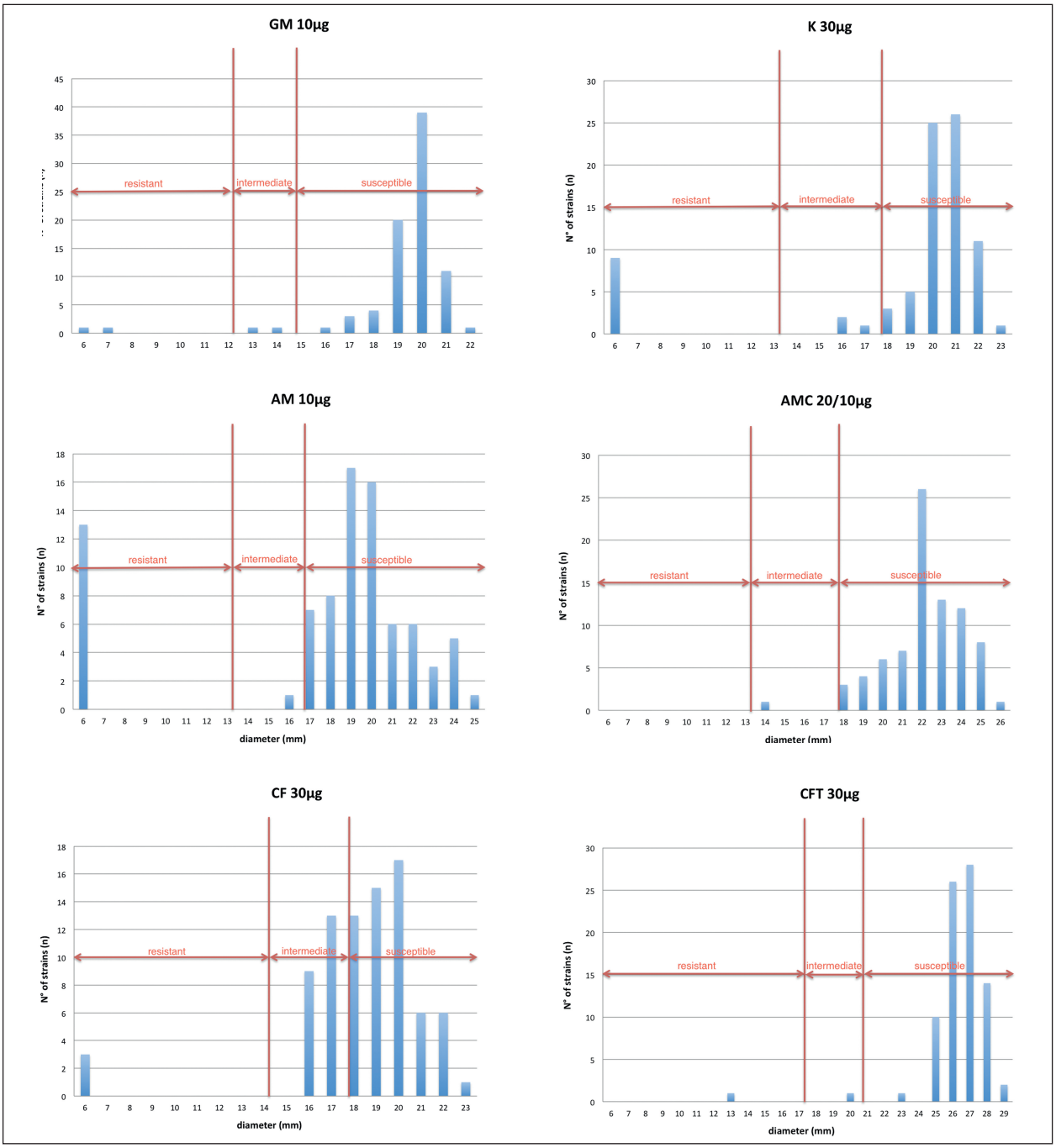

Figure 1: Distribution of inhibition zone diameters for the tested antimicrobial agents (GM: gentamicin; K: kanamycin; AM: ampicillin; AC: amoxicillin-clavulanic acid; CF: cephalothin; CFT: ceftiofur).

mastitis. The high genetic heterogeneity among the strains underlines that, in contrast to *S. aureus* (Stutz et al., 2011), no specific *E. coli* clones are mainly responsible for the *E. coli* mastitis situation.

This fact further highlights, that besides the use of antibiotics, the strategy for treatment of mastitis should be flanked by preventive measures. These comprise a number of approaches such as maintenance of a clean, dry, comfortable environment; proper milking procedures; proper maintenance and use of milking equipment; appropriate management of clinical mastitis during lactation; effective dry cow management; maintenance of biosecurity for contagious pathogens and culling of chronically infected cows as well as regular monitoring of udder health status.

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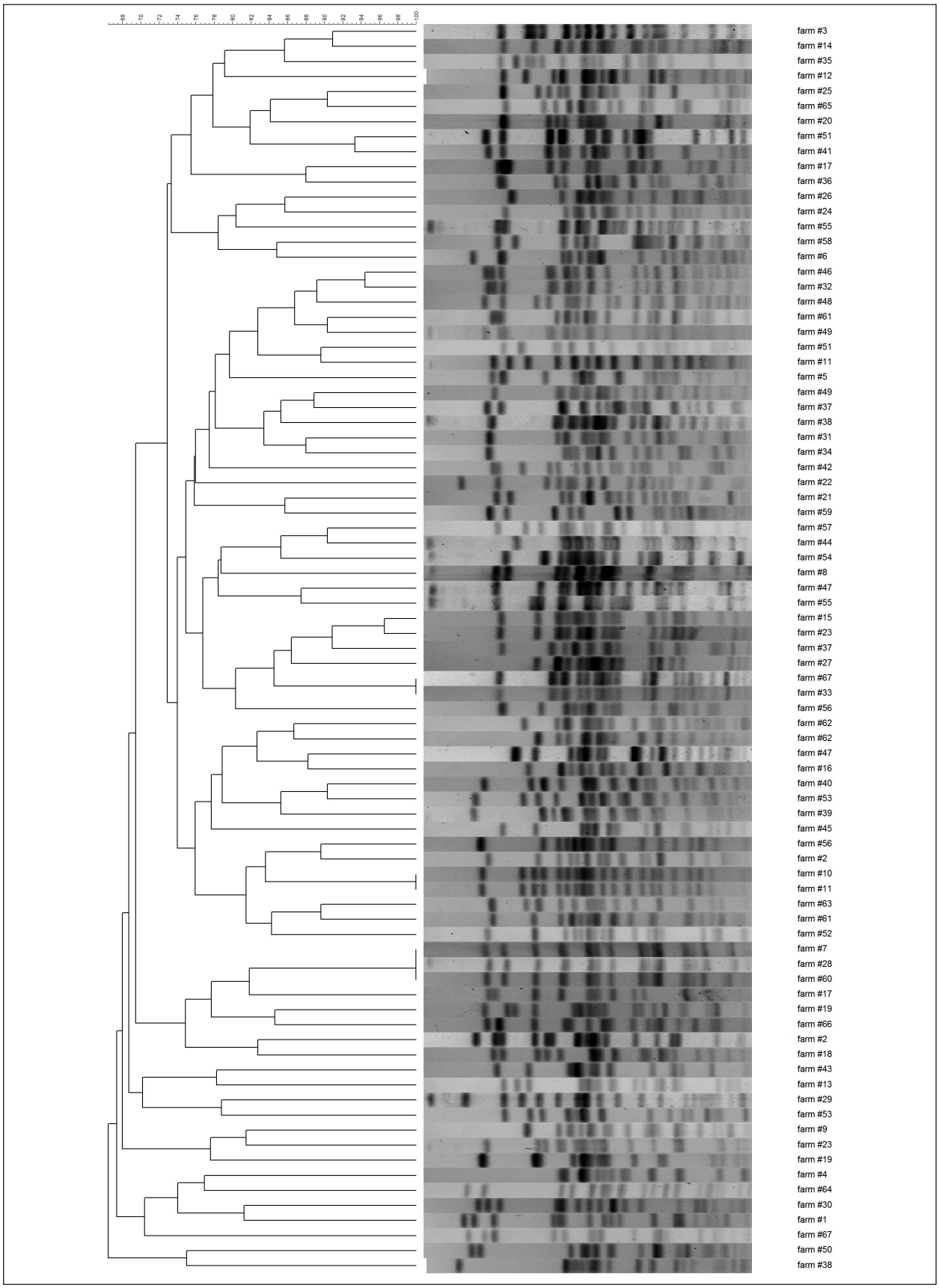

Figure 2: Cluster analysis and dendrogram of PFGE pattern of 83 *E. coli* isolates from mastitis milk samples. The dendrogram was generated using BioNumerics software GelCompare and Dice coefficient and UPGMA. Analysis parameters were set to 2 % for both optimization and tolerance values respectively. For clonal relationship, the cut off value (see line) was set at 95 %.

Profils de résistance et diversité génétique des souches d'*Escherichia coli* isolées à partir de mammites bovines aigües

Entre mars 2011 et février 2012, 83 souches d'*E. coli* issues de 83 vaches différentes provenant de 67 exploitations ont été collectées et testées quant à leur sensibilité vis-à-vis de divers antibiotiques. Ces antibiotiques ont été choisis sur la base de leurs autorisations pour l'application intra mammaire en Suisse et le test a été effectué par diffusion sur gel d'agar. En outre toutes les souches ont été typisées quant à leur appartenance aux groupes phylogénétiques. 16.9 % des souches présentaient une résistance à un ou plusieurs antibiotiques. L'amoxicilline-acide clavulanique, la gentamicine et les céphalosporines de troisièmes générations se montraient efficaces contre la majorité des souches d'*E. coli*. On a toutefois trouvé une souche fabriquant un extended-spectrum-beta-lactamase qui portait le gène *bla*$_{\text{CTX-M-14}}$. L'analyse génétique groupait la majorité des souches (87 %) dans les groupes phylogénétiques A et B1. La génotypisation par PFGE montrait une grande diversité entre les souches, même si elles provenaient de la même exploitation.

Profili di resistenza e variabilità genetica dei ceppi di *Escerichia coli* isolati nei bovini affetti da mastite acuta

Tra marzo 2011 e febbraio 2012 sono stati raccolti 83 ceppi di *E. coli* da 83 bovini differenti provenienti da 67 aziende e testati rispetto alla loro sensibilità ai diversi antibiotici. Gli antibiotici sono stai scelti in virtù dell'approvazione in Svizzera per l'applicazione intramammaria e il test sulla sensibilità è stato effettuato mediante il metodo di diffusione in agar. Inoltre tutti i ceppi sono stati fenotipizzati in rapporto alla loro appartenenza a gruppi filogenetici, nonché via electroforesi su gel a campo forzato (PFGE). Il 16.9% di tutti i ceppi hanno esibito resistenze a uno o più antibiotici. Amoxicillina e acido clavulanico, gentamicina e cefalosporine della terza generazione hanno manifestato un'efficacia contro la maggior parte dei ceppi di *E. coli*. Tuttavia, è stato trovato della beta-lattamasi a spettro esteso portatrice del gene *bla*$_{\text{CTX-M-14}}$. L'analisi genetica ha raggruppato la maggioranza dei ceppi (87%) nei gruppi filogenetici A e B1. Un'ulteriore genotipizzazione mediante PFGR ha mostrato una grande diversità tra i ceppi di *E. coli* anche se questi provenivano dalla medesima azienda.

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