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ANTIBIOTIC RESISTANCE IN *ENTEROBACTERIACEAE* STRAINS ISOLATED FROM CHICKEN AND MILK SAMPLES

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ABSTRACT

Antibiotic resistance and identification of strains in *Enterobacteriaceae* genera isolated from milk, milk products and rectal swabs of chicken was examined in this experiment. After samples collection cultivation and identification of bacterial strain was done. MALDI TOF MS Biotyper for identification of *Enterobacteriaceae* strains was used. For susceptibility testing disc diffusion methodology was used according by EUCAST. Results showed high level of ampicillin resistance in isolates from milk and milk samples. The highest streptomycin resistance was detected in isolates from rectal swabs of chicken. After identification, we determined that *S. enterica* ser. Typhimurium, which was isolated from rectal swabs of chicken showed the most multi-resistance from all identificated strains of *Enterobacteriaceae*. The most isolates bacterial strain was *E. coli*, which showed resistance against four antibiotics from rectal swabs of chicken. Also our results showed that the higher resistance level is in rectal swabs of chicken like in milk samples.

Keywords: Antibiotic resistance, *Enterobacteriaceae*, chicken, milk

INTRODUCTION

Antibiotic resistance is significant health, social and economic problem at this time. Antibiotic resistance of bacteria is biological risk, which increases morbidity and mortality of animal and human **(EFSA, 2008)**. **Keyser *et al.* (2008)** note that in recent years, accumulating problems with bacteria, which are resistant to antibiotics, leading to predictions that we return to the time before the discovery of antibiotics. Resistant bacteria from the intestines of food animals may be transferred to retail meat products resulting from fecal contamination during various stages of the slaughter process (e.g., evisceration) and subsequent handling of animal tissue **(Jackson *et al.*, 2001)**. Endogenous bacterial flora may play an important role as acceptor and donor of transmissible drug resistance genes **(Davies *et al.*, 1994 and Sunde *et al.*, 1998)**. The *Enterobacteriaceae* family is commonly used as an indicator of fecal contamination during food microbiological analyses, and includes important zoonotic bacteria such as *Salmonella* spp., *Yersinia* spp. and *Escherichia coli*. *Enterobacteriaceae* are the significant causes of serious infection, and many of the most important members of this family are becoming increasingly resistant to currently available antimicrobials **(Paterson, 2006)**. Recently, antimicrobial resistance has been reported in bacteria isolated from organic dairy products **(Sato *et al.* 2004; Sato *et al.* 2004; Tikofsky *et al.* 2003)**, and in poultry products related to *Salmonella* and *Campylobacter* **(Cui *et al.* 2005; Soonthornchaikul *et al.*, 2006)**. However, little information relative to commensal bacteria isolated from poultry meat and milk products is currently available. Consequently, the main goal of the present study was to investigate the prevalence of antimicrobial susceptibility found in *Enterobacteriaceae* isolates derived from chicken meat and milk products.

MATERIAL AND METHODS

Collection of samples

In 2010-2013, a total of 316 samples were collected from different animal farm from Slovakia. In total, 110 rectal swabs of and 206 samples of milk products (100 milk samples, 52 cheese samples, 28 sheep's whey samples and 26 sheep cheese samples). A total of 376 isolates of *Enterobacteriaceae* genera isolates were isolated in this research. Samples was collected by sterile swabs (Copan Inovation, Italy) and transported to the laboratory in transported medium.

Cultivation of microorganisms

Bacterial samples were spread on the surface of agar by sterile cotton swab directly. For cultivation of microorganism from *Enterobacteriaceae* genera selective media MacConkey agar was used. Cultivations were done at 37 °C at 24 hours for *Enterobacteriaceae* genera species in the aerobic condition. Recultivation after the first incubation was needed to obtain pure cultures of microorganisms at the same conditions. For purifying of microorganisms colonies and probably identification of *Enterobacteriaceae* strains Chromogenic coliform agar (Oxoid, UK) was used. For obtaining the pure culture of microorganisms four-ways streak plate method was used. Every recultivation steps were done in the same conditions.

Identification of microorganisms

Identification of microorganisms was done by several following methods. The firstly previously mentioned selective and chromogenic media for *Enterobacteriaceae* genera was used. Pure colonies of Enterobacteriaceae species was identified by MALDI TOF MS Biotyper (Brucker, Germany). Methodological procedure for preparing of samples and identification methods were done by **Kmeť and Drugdová, (2012)**.

Antibiotic susceptibility testing

After purifying of colonies, these were suspended in physiological solution inoculated from agar plates and every suspension were adjusted to equal a 0.5 McFarland standard density, where we used standardized densitometer (DensiLaMeter, ErbaLachema, Czech Republic). In this research following antibiotics were used: penicillins – ampicillin (10 µg/disc), piperacillin (30 µg/disc), cephalosporins – ceftriaxone (30 µg/disc), cefotaxime (5 µg/disc), carbapenems – doripenem (10 µg/disc), meropenem (10 µg/disc), fluoroquinolones – nalixid acid screen (10 µg/disc), levofloxacin (5 µg/disc), ofloxacin (5 µg/disc), aminoglycosides – gentamicicin (10 µg/disc), streptomycin (10 µg/disc), amikacin (30 µg/disc), tetracyclines – tigecycline (15 µg/disc), tetracycline (30 µg/disc) and miscallaneous agent represented by chloramphenicol (30 µg/disc). Antibiotic discs were purchased in OXOID, England. For susceptibility testing of *Enterobacteriaceae* species disc diffusion methodology was used according by **EUCAST, (2013a)**. Bacterial species was tested on Mueller-Hinton agar. Cultivation of bacterial species from

Enterobacteriaceae genera were done at 35±2 °C during 16-20 hours. Interpretation of inhibition zones around the disc were done according by breakpoint tables of **EUCAST, (2013b)**.

RESULTS AND DISCUSSION

In this study, we researched antibiotic resistance in bacteria from *Enterobacteriaceae* genera isolated from milk products and rectal swabs of chicken. We considered resistance in bacteria by disc diffusion methodology according by EUCAST. From the results, we determined that the most resistant *Enterobacteriaceae* strains was against natural and semi-natural antibiotics such as penicillins, aminoglycosides and tetracyclines. In this research *E. coli* strains were isolated the most often. *E. coli* is a main representative bacteria from *Enterobacteriaceae* genera **(Finney *et al*., 2003)**, therefore we noted them in this research as individual category. *E. coli* isolates isolated from previous mentioned samples were the most resistant against ampicillin (35.89 %), levofloxacin (20.51 %), streptomycin (20 %), piperacillin (16.07 %) and tetracycline (12.9 %). Smaller resistance *E. coli* showed against ofloxacin (4.16 %) and chloramphenicol (0.7 %) (see Table 1). Authors **Sáenz *et al.* (2001)** determined antibiotic resistance of *E. coli* in broiler chicken and they detected resistance against 88 % even, 40 % resistance against gentamicin, 58 % resistance against ampicillin, 15 % resistance against tetracycline and 12 % resistance against chloramphenicol. Higher resistance against antibiotics determined authors **Cho *et al*. (2012)**, who searched resistance in *E. coli* isolated from different husbandry and they determined resistance against ampicillin in 87 to 93 % level. Tetracycline resistance was detected in 58 to 60 %. Resistance to carbapenems was not detected like in our research. But they detected resistance against cefalotine, but didn't detected resistance to other cephalosporins like in our study. **Van *et al*. (2008)** isolated *E. coli* from different type of husbandry animal and chicken too. They detected resistance against ampicillin (20 – 84.2 %), tetracycline (60 – 100 %), gentamicin (0 – 47.5 %), nalixid acid (0 – 68.4 %) and chloramphenicol (20 – 65 %). The highest level of resistance was measured by **Lei *et al.* (2010)**, who determined that *E. coli* was from 64 to 100 % against ampicillin. Resistance against gentamicin (32.6 – 76.9 %), chloramphenicol (39.7 – 90.3 %), tetracycline (60.3 – 98.1 %). Many researchers examined antibiotic resistance of *E. coli* from different samples and everybody meats in one opinion. Antibiotic resistance of bacteria is differ from study to study **(Lira *et al*., 2004; Picozzi *et al*., 2005; Caro *et al*., 2007 and Čížek *et al*., 2007)**. Also we examined antibiotic resistance in *Enterobacteriaceae* strains isolated from milk and milk products, because its a products which people use to direct consumption. If products which contain antibiotic resistant bacteria, then these can transfer resistant genes from non-pathogenic to pathogenic bacteria. This fact can lead to problem with treatment of diseases caused by resistant bacteria **(Sorum and Sunde, 2001)**. Slightly different situation was determined in the samples of milk products. We determined that the most resistant from the all tested samples, in the case of ampicillin. Even 59 % of isolates showed resistance against ampicillin. Resistance against other used antibiotics were in the lower level. A 17.39 % of samples was resistant against tetracycline, 10.53 % were resistant against cefotaxime, 7.14 % were resistant against piperacillin and 3.13 % were resistant against chloramphenicol (Table 1). From the samples of milk and milk products were isolated following strains: *Escherichia coli*, *Serratia* spp., *Serratia rubidae*, *Serratia odorifera* bv. 1, *Serratia odorifera* bv. 2, *Serratia plymuthica*, *Klebsiella* spp., *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Citrobacter* spp., *Citrobacter braakii*, *Citrobacter gillenii*, *Enterobacter* spp., *Enterobacter aerogenes*, *Enterobacter omnigenus*, *Enterobacter kobei*, *Enterobacter georgoviae*, *Enterobacter cloacae*, *Yersinia* spp., *Raoultella ornithinolytica* a *Raoultella terrigena*. Resistance against ampicillin was detected in all isolated strains, expect *Citrobacter gilenii*. Resistance against three antibiotic together was determined in *E. coli*, *S. plymuthica* and *C. gilenii*. Resistance against two antibiotics was determined in *Serratia* spp. and *Serratia rubidae* and *Citrobacter* spp. More described results are in the Table 2. Also **Solomakos *et al*. (2009)** examined resistance in *E. coli* isolated from milk samples and they determined resistance against ampicillin, tetracycline, chloramphenicol. Their work showed resistance against streptomycin and gentamicin. **Farzana *et al*. (2009)** determined higher levels of resistance against ampicillin and chloramphenicol. Conversely, resistance against tetracycline was in lower level (less then 10 %). They isolated following bacteria: *E. coli*, *Enterobacter* spp. and *Klebsiella* sp.) Antibiotic resistance of isolates isolated from rectal swabs of chicken was different too. The main resistance was against streptomycin, even 86.36 %. Equally penicillins resistance was determined against ampicillin (31.25 %) and piperacillin (28.57 %). Resistance against tetracycline was 25 %, levofloxacin 5.55 %, chloramphenicol 4.5 % and resistance against ofloxacin 1.52 % (Table 1). **Miranda *et al*. (2008)** determined similar results as we in our study. They determined resistance against ampicillin (21.7 – 48.3 %), chloramphenicol (0 – 6.7 %) and gentamicin (0 – 5 %). Also **Machado *et al*. (2008) examined** resistance against tetracycline and streptomycin and they detected resistance too. The higher level of resistance determined **Tessi *et al*. (1997),** who noted in their study that resistance against tetracycline was from 49.3 to 52.7 % and streptomycin from 37.6 to 39.4 %. Similar results as **Tessi *et al*. (1997)** were detected by **Van *et al*. (2008)**. **Lei *et al*. (2010)** determined higher level of resistance as we in our study. From the rectal swabs of chicken we isolated following strains: *Escherichia coli*, *Serratia* spp., *Serratia fonticola*, *Citrobacter freundii*, *Enterobacter cloacae*, *Raoultella ornithinolytica*, *Proteus vulgaris*, *Shigella flexneri* a *Salmonella enterica* ser. Typhimurium. Also we examined resistance in these strains and we determined that the most resistant was *S. enterica* ser Typhimurium which was resistant to 5 antibiotic. *E. coli* and *Raoultella ornithinolytica* were resistant against four antibiotics. Resistance against two antibiotics showed in *E. cloacae*. *Serratia fonticola* was resistant against ampicillin only and *Shigella flexneri* against chloramphenicol only. Results are more described in the Table 3. Many researchers confirmed multi-resistant strains isolated from rectal swabs of chicken **(Unno *et al*. 2010; Lim *et al*. 2007; Miranda *et al*. 2008)**. Authors **Husseina *et al*. (2008)** identificated bacteria as *E. coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus* spp., *Proteus mirabillis* and *Pseudomonas aeruginosa*. They detected resistance against antibiotic which we didnt used in this experiment. Also they detected multi-resistant *E. coli*. **Miranda *et al*. (2008)** identificated bacteria as *Serratia* spp., *Enterobacter* spp., *Klebsiell*a spp. and *Escherichia* spp. and they detected resistance against ampicillin, cholramphenicol and gentamicin.

Table 1 Resistance of *Enterobacteriaceae* genera and *E. coli* isolated from milk products and chicken

Antimicrobial agents (μg)	*Enterobacteriaceae*											
	E. coli only				Milk products				Chicken			
	N	R	I	S	**N**	R	I	S	**N**	R	I	S
Penicillins												
Ampicillin (10)	**117**	35.89	ND	64.11	**100**	59	8	33	**96**	31.25	3.12	65.63
Piperacillin (30)	**56**	16.07	ND	83.93	**14**	7.14	0	92.86	**28**	28.57	7.14	64.29
Cephalosporins												
Ceftriaxone (30)	**24**	0	ND	100					**66**	0	0	100
Cefotaxime (5)	**18**	0	ND	100	**19**	10.53	24.32	63.16				
Carbapenems												
Doripenem (10)	**18**	0	ND	100					**18**	0	0	100
Meropenem (10)	**24**	0	ND	100	**19**	0	0	100	**66**	0	0	100
Fluoroquinolones												
Nalixid acid (10) screen	**14**	0	ND	100	**32**	0	9.38	90.62				
Levofloxacin (5)	**39**	20.51	ND	79.49	**6**	0	0	100	**18**	5.55	0	94.45
Ofloxacin (5)	**24**	4.16	ND	95.84					**66**	1.52	0	98.48
Aminoglycosides												
Gentamicin (10)	**43**	0	ND	100	**28**	0	0	100	**43**	0	2.33	97.67
Streptomycin (10)	**20**	20	ND	80	**31**	0	9.68	90.32	**22**	86.36	0	13.63
Amikacin (30)	**64**	0	ND	100	**15**	0	0	100	**43**	2.33	0	97.67
Tetracyclines												
Tigecycline (15)	**56**	0	ND	100					**28**	0	0	100
Tetracycline (30)	**62**	12.9	ND	87.1	**46**	17.39	4.35	78.26	**64**	25	11	64
Miscallaneous agents												
Chloramphenicol (30)	**142**	0.7	ND	99.3	**96**	3.13	0	96.87	**110**	4.5	0	95.5

Legend: N – number of tested strains, R – percentage of resistance, I – percentage of intermediate isolates, S – percentage of susceptible strains, ND – not detected

Table 2 *Enterobacteriaceae* isolates from milk and milk products, sources and antibiotic resistance profile

Microorganisms	Source of isolates	Antibiotics
Escherichia coli	Sheep whey, cheeses, milk	AMP, PIP, TET
Serratia spp.	Milk, cheese	TET, AMP
Serratia rubidea	Milk	CHL, AMP
Serratia odorifera bv. 1	Smoked cheese	AMP
Serratia odorifera bv. 2	Cheese	AMP
Serratia plymuthica	Milk	TET, CHL, AMP
Klebsiella spp.	Milk, cheese, smoked cheese	AMP^{NR}
Klebsiella oxytoca	Smoked cheese, cheese	AMP^{NR}
Klebsiella pneumoniae	Cheese	AMP^{NR}
Citrobacter spp.	Milk, sheep whey	AMP, CHL
Citrobacter braakii	Cheese	AMP
Citrobacter gillenii	Sheep whey	PIP, CTX, CHL
Enterobacter spp.	Milk	AMP
Enterobacter aerogenes	Sheep whey, milk	AMP^{NR}
Enterobacter omnigenus	Milk	AMP
Enterobacter kobei	Smoked cheese	AMP
Enterobacter georgoviae	Sheep whey	AMP
Enterobacter cloacae	Milk	AMP^{NR}
Yersinia spp.	Milk	AMP
Raoultella ornithinolytica	Cheese, sheep whey, milk	AMP
Raoultella terrigena	Sheep whey, cheese	AMP

Legend: ^{NR} – natural resistance

Table 3 Enterobacteriaceae isolates from chicken and antibiotic resistance profile

Microorganisms	Antibiotics
Escherichia coli	AMP, TET, PIP, OFL
Serratia spp.	Susceptible
Serratia fonticola	AMP
Klebsiella oxytoca	AMP^{NR}
Citrobacter freundii	AMP^{NR}
Enterobacter cloacae	AMP^{NR} STR, TET
Raoultella ornithinolytica	AMP, STR, TET, CHL
Proteus vulgaris	AMP^{NR}
Proteus mirabilis	TET^{NR}
Shigella flexneri	CHL
Salmonella enterica ser. Typhimurium	AMP, PIP, LVX, OFL, CHL, TET

Legend: ^{NR} – natural resistance

CONCLUSION

Results from this study showed that the most resistance was against penicillins and aminoglycosides. The highest level of ampicillin resistance was determined in milk and milk samples. Conversely, the highest level of streptomycin resistance was detected in bacteria isolated from rectal swabs of chicken. Also these results showed that more multi-resistant strains were isolated from rectal swabs of chicken, where we determined that *Salmonella enterica* ser. Typhimurium was resistant against five antibiotics, then *E. coli* and *R. ornithinolytica* which were resistant against four antibiotics together. In the isolates from milk and milk products samples we isolated bacteria as *E. coli*, *S. plymuthica* and *C. gilenii* which were resistant against three antibiotics together. Other isolated and identificated bacteria showed resistance against maximum two or one antibiotics.

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