Staphylococcus and *Enterococcus* strains of water and food origin and their susceptibility to antibiotics

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Summary

Two hundred samples of foodstuffs and water were examined for the presence of *Enterococcus* sp., of which 105 were identified as positive. Sixty samples of foodstuffs and water were examined for the presence of *Staphylococcus* sp., of which 48 were identified as positive. The isolated strains were identified and, based on their generic attributes, they were ranked to genus *Enterococcus* and *Staphylococcus.* For sorting, a STAPHYtest 16, API Staph and EN-COCCUS tests were used. The isolates were further subjected to screening of susceptibility to antibiotics, a broth microdilution method was applied.

Keywords

Enterococcus; *Staphylococcus*; teicoplanin; vancomycin; susceptibility to antibiotics

Staphylococcus species commonly reside on animal and human skin and its near surroundings, from which it may enter the food. Hence, food chain might be a likely source of staphylococci contamination in humans, particularly endangered are salted food, hams, eggs, confectioneries and etc. *Staphylococcus* was frequently isolated from raw and processed red meat as well as from chicken carcasses [1].

Staphylococci could be the cause of a variety of infections. Toxigenic strains of *St. aureus* are the most important ones causing enterotoxicosis and toxic shock syndrome. The coagulase negative staphylococci, which are generally considered as bacteria of low pathogenicity, have been found as causative agents of severe or even lethal infections in persons with special predisposition (*e.g.* immunocompromised patients in oncology, haematology, nephrology, and transplantation units). Multiresistant endemic strains of S*taphylococcus* species are particularly dangerous, being involved in nosocomial infections [2]. If food contaminated with coagulase negative S*taphylococcus* species is stored under conditions that may promote growth of these species, alimentary diseases could be induced [3].

In recent years, new species and subspecies have been described, and a number of infections have increased. Increasing resistance to antibiotics was found in many S*taphylococcus* isolates. For example, resistant species were isolated from cheese made from raw milk and from raw meat products. Different antibiotic resistance patterns including resistance to tetracycline, erythromycin, chloramphenicol, penicillin and kanamycin were identified among these isolates [4]. In 1996, methicillin resistant, coagulase negative S*taphylococcus* was isolated from young and healthy chickens' nares and skin [5].

Enterococci are Gram-positive bacteria that live as a part of the natural flora in the intestinal tract of animals and humans. Soil, surface and sewage water, surface of plants and vegetables may also contain *Enterococcus* species. The isolation of *Enterococcus* sp. has often been used to indicate faecal contamination of water [6], and could indicate the presence of enteropathogenic bacteria, such as species of *Staphylococcus* and *Listeria* [7]. Enterococci are considered as low pathogenic species. In immunocompromised individuals, they may cause severe diseases such as urinary tract infections, endocarditis or meningitis. A range of

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natural and acquired antibiotic resistance could hinder antibiotic treatment of infectious diseases. Vancomycin-resistant enterococci represent a significant problem as they can produce nosocomial infections.

The aim of this study was to evaluate occurrence of enterococci and staphylococci in foodstuffs and in water and to determine their resistance to antibiotics.

MATERIAL AND METHODS

Detection and identification of *Staphylococcus* sp.

Well water samples were collected in sterile screwed-cap glass bottles, stored in refrigerator, and analyzed within 24 h of sample collection. Food samples were bought in retail outlets and stored in refrigerator until used. Food samples were analyzed according to ČSN EN ISO 6886 part 1 [8] using Baird-Parker selective agar plate (HiMedia, Bombay, India). 100 ml aliquots of water sample were concentrated and filtered through 0.45 μm pore size membrane filters (Pall-Gelman, Ann Arbor, USA). The filters were placed onto Baird-Parker selective agar plates. Gram-positive cocci were transferred onto blood agar plate and nutrient agar no. 2 (Himedia, Bombay, India). The genus *Staphylococcus* was confirmed using the oxidase test, catalase test, plasmacoagulase test, colony appearance on Baird-Parker agar plates, haemolysis behaviour and pigment formation. The species were determined using commercial biochemical sets (STAPHYtest 16, Pliva-Lachema, Brno, Czech Republic and API Staph test, BioMérieux, Lyon, France) and identification software TNW lite vs. 6.0 (Pliva-Lachema, Brno, Czech Republic) and APILAB (BioMérieux, Lyon, France). Confirmed *Staphylococcus* strains were maintained on blood agar plates in refrigerator and were transferred monthly.

Detection and identification of *Enterococcus* sp.

The presence of *Enterococcus* sp. in food was determined by a conventional procedure - plating onto Slanetz-Bartley selective agar plates. Membrane filtration technique according to ČSN EN ISO 7899-2 [9] was applied to isolate enterococci from water samples. In order to discern the accompanying microflora grown on M-F-Enterococcus Selective Agar plates (Merck, Darmstadt, Germany), suspected colonies were subjected to Gram staining. Gram-positive cocci were inoculated onto blood agar plates, and the presence of catalase and pyrrolidonylarylamidase was performed using the PYR test (ITEST plus, Hradec Králové, Czech Republic). In the case of catalase negative and pyrrolidonylarylamidase positive colonies, the genus *Enterococcus* was tested for growth in nutrient broth no. 1 with 6.5% NaCl, in alkali broth (nutrient broth no. 1, pH = 9.6 - HiMedia, Bombay, India), in broth with 40% bile (nutrient broth no. 1 with 40% bile - HiMedia, Bombay, India), and for growth at 43 °C and esculine hydrolysis. Species were determined by examining the series of hydrocarbon utilizations and by using a commercial test (EN-COCCUStest, Pliva-Lachema, Brno, Czech Republic). Confirmed *Enterococcus* species were maintained on blood agar plates at refrigerator temperature.

Susceptibility to antibiotics

Cell suspension of enterococci and staphylococci was prepared using 0.5–1.0 McFarland turbidimetric standards. This suspension was inoculated in microtitrate plates with antibiotics using dispenser. The Minimal Inhibition Concentration (MIC) values were determined after 24 h of incubation at 37 °C, and then were compared with the MIC values recommended by the National Reference Laboratory for Antibiotics [10]. A strain was found to be resistant to antibiotic if the determined MIC value was above the recommended value. Penicillin resistance was confirmed by detection of β-lactamase, the enzyme cleaving chromogenic cefalosporine (NITROCEFIN, Oxoid, Hampshire, United Kingdom).

RESULTS AND DISCUSSION

Detection and identification of *Staphylococcus* sp.

In total, 46 food samples and 14 well water samples were collected and the presence of *Staphylococcus* species determined. The selection of food samples focused on those, which are at high risk of being contaminated with staphylococci; salted cheese, soft salami, confectioneries, products containing eggs and delicatessen. Some samples were contaminated by 1 up to 4 different species. From 60 food and water samples, 80% (48 samples) were positive for *Staphylococcus* species, in total fifteen species being identified. The incidence varied from one to four species per sample. Isolated staphylococci belonged to one of the following species: *St. warneri* (17 strains), *St. saprophyticus* subsp. *saprophyticus* (11 strains) and *St. epidermidis* (6 strains). Coagulase-positive *St. aureus* subsp. *aureus* and *St. aureus* subsp. *anaerobicus* were also isolated from four and two samples, respectively (Fig. 1).

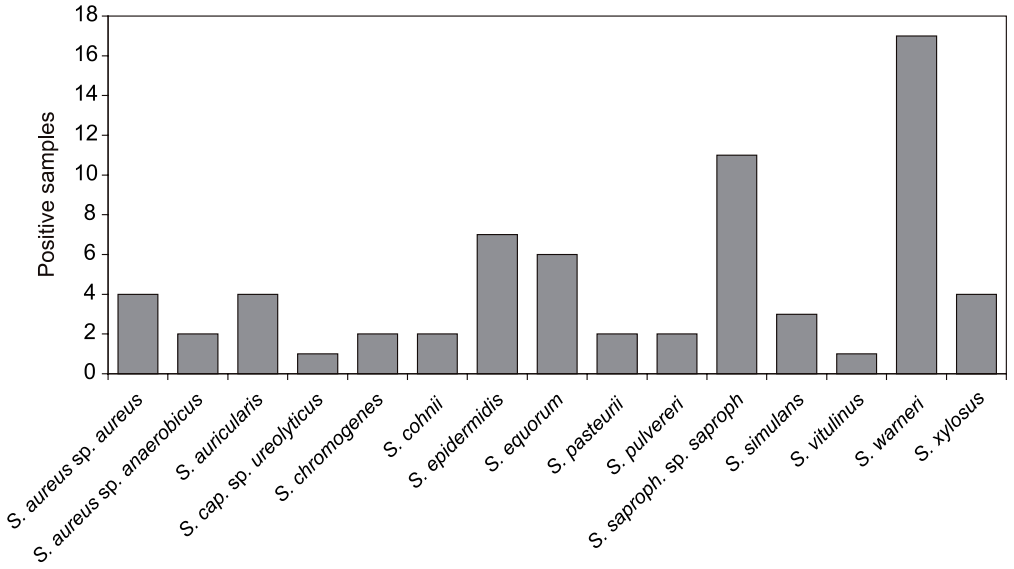

Fig. 1. Occurrence of *Staphylococcus* sp. in food and well water samples.

Tab. 1. Incidence of *Staphylococcus* sp. in food and water samples.

Sample	Number of samples (positive/total)	*Staphylococcus* strain														
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Well water	8/14	0	0	0	1	0	0	0	1	0	0	3	0	0	5	0
Meat products																
Salami Junior	3/4	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0
Gothai salami	3/4	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0
Sausage	3/3	0	0	0	0	1	0	1	0	0	0	0	0	0	1	2
Debrecen meat	1/1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0
Liver pate	2/3	0	0	2	0	0	1	0	0	0	0	1	0	0	0	0
Chicken wing – swab	2/2	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Meal	2/2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Delicacy products																
Egg salad	2/2	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0
Open sandwich	2/2	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0
Ham foam	2/2	0	0	0	0	0	0	0	2	0	0	0	0	1	2	0
Dessert	3/3	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1
Obložené vejce♣	1/1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
Egg in aspic	2/2	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0
Salted cheeses																
Olomoucké tvarůžky♦	3/3	0	0	0	0	0		1	0	0	0	2	0	0	0	1
"Korbačiky"♥	3/3	0	0	0	0	0	0	2	0	1	0	1	0	0	1	0
Other food products																
Raw milk	2/2	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0
Milk powder	1/1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Egg whisky	0/1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mustard – "Kremž"	1/1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Mustard – Oriental	0/1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vegetable	1/1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Yeast extract	1/1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	48/60	4	2	4	1	2	2	7	6	2	2	11	3	1	17	4

♣ - egg with ham and salad with mayonnaise; ♦ - soft ripened cheese; ♥ - steamed cheese.
A - *S. aureus* sp. *aureus*, B - *S. aureus* sp. *anaerobicus*, C - *S. auricularis*, D - *S. cap.* sp. *ureolyticus*, E - *S. chromogenes*, F - *S. cohnii*, G - *S. epidermidis*, H - *S. equorum*, I - *S. pasteurii*, J - *S. pulvereri*, K - *S. saproph.* sp. *saproph*, L - *S. simulans*, M - *S. vitulinus*, N - *S. warneri*, O - *S. xylosus*.

An overview of isolated *Staphylococcus* strains in respect to their origin is listed in Table 1. Higher prevalence of staphylococci was found in meat products (73.3%) than it was in the well water samples (57.1%). Food processing (e.g. cutting, packaging in retail outlets) could be considered as a source of *Staphylococcus* contamination [11]. Each swab sample of chilled chicken wing contained *Staphylococcus* species. There are a lot of critical points during the poultry processing, in which secondary contamination took place. High incidence of staphylococci in food is also caused by their insusceptibility to pH changes and higher salt contents, so that some food preservation techniques may be inadequate. Staphylococci were isolated from all tested delicatessen products (100%) including those containing eggs (e.g. open sandwich, egg salad, egg in aspic), ham foam and desserts. A high degree of manual operation during processing as well as working equipments might be the likely source of contamination. Staphylococci are also able to persist on the surface of devices by forming a biofilm [12]. All tested salted cheeses also harbored *Staphylococcus* species, probably due to the ability of the latter to survive at presence of 10% NaCl. Cheeses are likely to be contaminated with *Staphylococcus* during processing, distributing and selling in markets; therefore successful isolation of staphylococci has been frequently recorded [13]. The occurrence of *Staphylococcus* in milk powder, pasteurized milk and mustard was probably due to secondary contamination, since these products were temperature treated.

Susceptibility to antibiotics

Minimal inhibitory concentration (MIC) values were determined using the microbroth diluting method and the results are summarized in Table 2. All of the analyzed strains of *Staphylococcus* were sensitive to chloramfenicol, sulphamethoxazol/trimethoprim, ciprofloxacin, teicoplanin and vancomycin. Sixty-five percent of isolated strains were resistant to penicillin, and 37% were resistant to erythromycin. One strain of *St. saprophyticus* subsp. *saprophyticus* showed susceptibility to oxacillin similar to the recommended standard, so that it was not indicated as a resistant strain. From 17 strains of *St. warneri*, 13 were found to be resistant to erythromycin. From food samples in this study, no multiresistant *Staphylococcus* strain was isolated.

Surprisingly, *Staphylococcus* species acquire resistance to the newly developed antibiotics very rapidly. Although resistant *Staphylococcus* strains predominane in the isolates of clinical and veterinary origin, food could also serve as a significant source of antibiotic resistant staphylococci. Occurrence and spread of antimicrobial resistance

Tab. 2. Antibiotic resistence patterns of *Staphylococcus* sp. isolated from food and well water samples.

Species	Number of samples	A number of resistant strains of *Staphylococcus* isolates											
		PEN	OXA	AMS	CMP	TET	COT	ERY	CLI	CIP	GEN	TEI	VAN
A	4	1	0	0	0	0	0	1	0	0	0	0	0
B	2	2	0	0	0	0	0	1	0	0	0	0	0
C	4	2	0	0	0	1	0	1	0	0	0	0	0
D	1	1	0	0	0	0	0	0	0	0	0	0	0
E	2	2	0	0	0	0	0	0	0	0	0	0	0
F	2	1	0	0	0	0	0	1	0	0	0	0	0
G	7	3	0	0	0	0	0	5	0	0	0	0	0
H	6	3	0	0	0	0	0	0	0	0	0	0	0
I	2	1	0	0	0	0	0	0	0	0	0	0	0
J	2	1	0	0	0	0	0	1	0	0	0	0	0
K	11	8	1	1	0	0	0	2	0	0	1	0	0
L	3	2	0	0	0	0	0	0	0	0	0	0	0
M	1	0	0	0	0	0	0	0	0	0	0	0	0
N	17	15	0	0	0	0	0	13	1	0	0	0	0
O	5	3	0	0	0	0	0	0	0	0	0	0	0
Total	67	45	1	1	0	1	0	25	1	0	1	0	0

A - *S. aureus* sp. *aureus*, B - *S. aureus* sp. *anaerobicus*, C - *S. auricularis*, D - *S. cap.* sp. *ureolyticus*, E - *S. chromogenes*, F - *S. cohnii*, G - *S. epidermidis*, H - *S. equorum*, I - *S. pasteurii*, J - *S. pulvereri*, K - *S. saproph.* sp. *saproph*, L - *S. simulans*, M - *S. vitulinus*, N - *S. warneri*, O - *S. xylosus.*

PEN - benzylpenicillin, OXA - oxacillin, AMS - ampicillin/sublactam, CMP - chloramphenicol, TET - tetracycline, COT - sulphamethoxazol/trimethoprim, ERY - erythromycin, CLI - clindamycin, CIP - ciprofloxacin, GEN - gentamicin, TEI - teicoplanin, VAN – vancomycin.

Tab. 3. Incidence of *Enterococcus* sp. in food and water samples.

Samples	Number of samples (positive/total)	*Enterococcus* strains				
		P	Q	R	S	T
Water samples						
Drinking well water	19/52	6	8	4	0	1
City distribution center	0/1	0	0	0	0	0
Supply water	2/2	1	1	0	0	0
Meat and meat products						
Salami	21/57	1	20	0	0	0
Meat pate	1/1	1	0	0	0	0
Ground meat	4/9	4	0	0	0	0
Pork offal	5/6	2	2	0	1	0
Wine sausage	5/8	3	2	0	0	0
Pork sausage	1/3	0	1	0	0	0
Pork brawn	1/1	0	1	0	0	0
Chicken offal	4/5	3	1	0	0	0
Chicken cavity swab	10/11	3	7	0	0	0
Chicken meat	6/8	4	2	0	0	0
Rabbit meat	1/4	1	0	0	0	0
MPP swab	6/9	3	3	0	0	0
Milk and milky products						
Milk powder	9/9	1	8	0	0	0
Whey powder	5/5	1	2	0	0	2
Raw milk	4/4	2	2	0	0	0
Processed cheese	0/1	0	0	0	0	0
Ice cream	1/1	0	1	0	0	0
Other food products						
Fish salad	0/1	0	0	0	0	0
Tartare sauce	0/1	0	0	0	0	0
Ground spices	0/1	0	0	0	0	0
Total	105/200	36	61	4	1	3

MPP - meat processing plant. P - *E. faecalis*, Q - *E. faecium*, R - *E. casseliflavus*, S - *E. mundtii*, T - *E.* species.

among species in environment represent a complex process, in which several factors are involved. To solve this widespread challenge, a multidisciplinary approach is called for.

Detection and identification of *Enterococcus* sp.

From the total of 200 samples we isolated 105 strains of enterococci, which belonged to the following species: 36 *E. faecalis*, 61 *E. faecium*, 4 *E. casseliflavus*, 1 *E. mundtii* and 3 strains could not be identified. The occurrence of enterococci in samples is given in Table 3; Fig. 2 shows the distribution of strains. In 21 out of 55 water samples *Enterococcus* strains were identified, namely: 7 *E. faecalis*, 9 *E. faecium*, 4 *E. casseliflavus* and one unidentified strain. Our results are in accordance with findigs of Švec and Sedláček [14], who reported high prevalence of *E. faecalis* and *E. faecium* strains in water. Of 57 salami samples, twenty one *Enterococcus* strains were isolated. *E. faecium* was the predominant species (20 strains), and

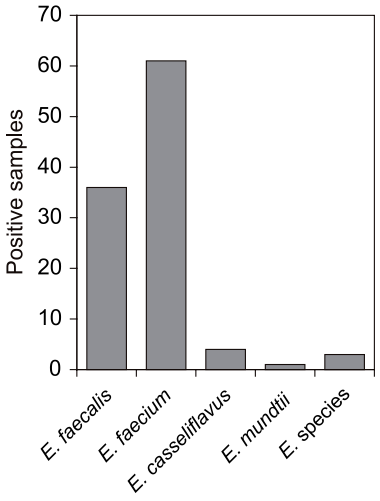

Fig. 2. Occurrence of *Enterococcus* sp. in food and well water samples.

Tab. 4. Antibiotic resistance patterns of enterococci isolated from food and water samples.

Species	Number of strains	A number of resistant strains of *Enterococcus* isolates											
		PEN	AMP	AMS	CLT	CMP	TET	ERY	CLI	VAN	TEI	FUR	OFL
P	36	35	0	0	33	0	7	6	31	0	0	6	12
Q	61	58	5	6	60	5	6	16	47	2	0	47	49
R	4	4	0	0	4	0	0	2	4	0	0	1	4
S	1	1	0	0	1	0	0	0	1	0	0	0	0
T	3	3	0	0	3	0	0	0	3	0	0	2	2
Total	105	101	5	6	101	5	13	24	86	2	0	56	57

P - *E. faecalis*, Q - *E. faecium*, R - *E. casseliflavus*, S - *E. mundtii*, T - *E.* species.
PEN - benzylpenicillin, CMP - chloramphenicol, VAN - vancomycin, AMP - ampicillin, TET - tetracycline, TEI - teicoplanin, AMS - ampicillin + sublactam, ERY - erythromycin, FUR - nitrofurantoin, CLI - clindamycin, OFL - ofloxacin.

E. faecalis was isolated only in one occasion. Thirty-eight *Enterococcus* strains were found in 56 meat samples. The following strains were confirmed: 21 *E. faecalis*, 16 *E. faecium*, 1 *E. mundtii*. Six strains of *Enterococcus* were isolated from 9 swab samples obtained from meat processing plant including 3 *E. faecalis* and 3 *E. faecium*. Incidence of enterococci in the meat processing plant could be explained by transferring cocci from gastro-intestinal tract during evisceration [6]. Dairy products (20 samples), tartare sauce, fish salad and ground spices contained *E. faecalis* (4 strains), *E. faecium* (13 strains), and 2 strains were not identified. Susceptibility to antibiotics is summarized in Table 4.

Susceptibility to antibiotics

All the investigated strains of *Enterococcus* are sensitive to teicoplanin and vancomycin. Low resistance (4 $mg.l^{-1}$) to vancomycin was observed in two strains isolated from a well water sample and milk powder, however these could not be considered as vancomycin resistant. Nevertheless, higher MIC value for vancomycin may indicate a possible incidence of truly vancomycin-resistant strains. In both cases, *E. faecium* was confirmed. In contrast to our results, Kolář et al. [15] found three strains of vancomycin-resistant *E. faecium* and one strain of vancomycin-resistant *Enterococcus* sp., which were recovered from 561 samples of animal origin and from 120 chicken cloacal swab samples.

Five strains (4.8%) of *Enterococcus* species were ampicillin and chloramphenicol resistant, six strains (5.7%) were found to be resistant to ampicillin/sublactam. Fifty-six (53.3%) *Enterococcus* strains were resistant to nitrofurantoin, and 67 strains (63.8%) to ofloxacin. These results indicate a low efficiency of nitrofurantoin and ofloxacin as antimicrobial agents. Enterococci possess natural resistance to penicillin, cefalotin and clindamycin. A major role in treating enterococcus infections in humans is attributed tothe development of new antimicrobial agents, such as streptogramines, glycopeptides, evernicomycines and oxazalididones.

CONCLUSION

E. faecium (58.1%) and *E. faecalis* (34.3%) belong to the most widespread. Other isolates contained *E. casseliflavus* (3.3%) and *E. mundti* (0.95%). The most frequently isolated strains of staphylococci were *S. warneri* (25.4%), *S. saprophyticus* (16.4%) and *S. epidermidis* (10.4%).

It was observed that the tested strains of enterococci showed the highest susceptibility to teicoplanin (100%) and vancomycin (98.1%), five strains (4.8%) were resistant to both ampicillin and chloramphenicol. As far as staphylococci are concerned, they were found highly sensitive to both teicoplanin and vancomycin (100%). There was resistance to benzylpenicillin and erythromycin, with an incidence of 63.5% and 44.9% respectively.

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