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Original Research Paper

BACTERIAL CONTAMINATION OF SHELLFISH IN SLOVENIA

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Summary: In 2003 and 2004 we examined 182 shellfish samples according to the present applicable rules. The samples were tested for the presence of *Salmonella* and enumeration of *Escherichia coli* with the methods recommended by European Community. More then 90 % of samples fulfilled the veterinary conditions about placing live shellfish on the market, concerning the level of 230 *E. coli* in 100 g of flesh. We detected the presence of *Salmonella* at 0.5 % of samples. That represents a rare but possible occasional appearance of this bacteria in shellfish. Concerning other contaminants that may cause poisoning by shellfish consumption, like pathogenic strains of *Vibrio parahaemolyticus*, *Norovirus* or hepatitis A virus, a lot of studies have been done, in a view to prepare new microbiological criteria for foodstuffs in Europe. It is now well recognised that bacterial indicators of faecal pollution (*E. coli* and faecal coliforms) do not adequately indicate the presence of enteric viruses. Another problem is that depuration process is more effective in removing *E. coli* and coliforms than viruses from shellfish.

Key words: food contamination; shellfish - microbiology; food analysis - methods; legislation, food

Introduction

Harvesting and shellfish consumption is an actual problem in Europe concerning seafood safety. Shellfish can be treated as risky food with high probability of poisoning. Bio – toxins have occurred occasionally in shellfish tissue as result of accumulation when growing and feeding in water containing high levels of toxin – producing plankton or pathogenic micro – organisms.

The Slovenian Rules on the veterinary conditions for the production and placing on the market of live shellfish (16) are based on the Directive 91/492/EEC of the European Community and entered into force in January 2004. The Rules set the veterinary conditions for the production and placing on the market of live shellfish intended for direct human consumption or for further processing (purification procedure) before use. Shellfish must fulfil microbiological criteria and criteria about containing toxins. Shellfish must also meet the requirements concerning the content of radionuclides and the presence of toxic and other disputable components appearing naturally or added to the environment, the intake of which could exceed the admissible daily intake, or which could deteriorate the taste of the molluscs (8).

The criteria shellfish must fulfil before marketing are the level of fecal coliform microorganism below 300/100 g of flesh and the level of *E. coli* below 230/100 g of flesh, and should not contain salmonellas in 25 g of flash. Shellfish with higher level of these bacteria can be put on the market after depuration in order to reach acceptable limits (16).

In the Rules on the veterinary-sanitary control of food production establishments, veterinary-sanitary checks and the conditions for health suitability of foodstuffs and raw materials of animal origin, in force from February 2000 (15) until the adoption of the above mentioned Rules, both criteria applied to live shellfish.

The prescribed method for the determination of *E. coli* and Coliform bacteria is determination of their levels by means of the most probable number (MPN) method, with five test tubes and triple dilution. The European Community recommends the modified method according to Donovan (5), based on the determination of the emergence of acids from the lactose.

The Directive (8) recommends the ISO 6579 method for the determination of the presence of salmonellas.

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Material and methods

In 2003, we examined 66 shellfish samples according to the Rules applicable at that time (15). In 2004 we examined 116 samples altogether, 6 samples before and 110 samples after the Rules on the veterinary conditions for the production and placing of live shellfish on the market came into force (16).

All samples were tested for *Salmonella spp.* and *E. coli* level, 6 samples were tested for the coliform count. The origin (harvested in Slovenia or imported) and the time of sampling (before or after depuration) of the shellfish varied.

Detection of Salmonella spp.

The presence of salmonellas in shellfish was determined in accordance with the recommended EU method, i.e. in line with the ISO 6579 standard (12).

The presence of salmonellas was determined in 25 g of whole flesh. The prepared samples were pre-enriched by means of 9-times the amount of peptone water (Buffered peptone water; Biolife, Milano, Italy) and incubated for 16 - 20 h at 37 °C. Afterwards, the samples were subcultured to two liquid enrichment media: Rappaport - Vassiliadis soya broth or RVS (Biokar, Allonne, France) and Mueller Kaufman tetrationat with novobiocin (MKTTn) (Biokar). The first medium was incubated at 37 °C, and the second at 41.5 °C for 24 h. From the enrichment media samples were subcultured to the selective media xylose lysine desoxycholate agar XLD (Merck, Dornstadt, Germany) and Rambach agar (Merck). The plates were incubated for 24 h at 37 °C. After incubation, the plates were examined for the presence of any characteristic colonies. If they were found, the biochemical and serologic confirmations followed.

Enumeration of Escherichia coli

From two options set by the Rules (16), we decided to determine the level of *E. coli* as recommended by the EU expert committee for this area.

The number of *E. coli* bacteria was determined by the method recommended by the EU Central Reference Laboratory (CRL) responsible for the area of microbiological contamination of the shellfish (CEFAS, Weymuth Laboratory, UK).

The samples were prepared according to the standard procedure for the preparation of stock dilutions (ISO 6887-2) and the examination was conducted according to the ISO/TC 34/SC 9 N 587 method (modified Donovan's method) (5).

In order to determine the number of *E. coli* bacteria, the primary enrichment medium of minerals – modified - glutamate broth - MMGB (Oxoid, Basingstoke, England) was used. From three consecutive dilutions (10^{-1}, 10^{-2}, 10^{-3}), one millilitre of the sample was inoculated into five test tubes containing the medium. By using these dilutions, the method can be used for the determination of the *E. coli* count higher than 200 cfu/100 g. To register lower level contamination (over than 20 cfu/100 g), 10 millilitres of the sample from the 10^{-1} dilution was inoculated into an additional series of five test tubes with MMGB. This way, 1 g of the sample was applied to each of them. The test tubes were incubated for 24 h at 37 °C and then the visible as a change in the colour of the medium was observed decomposition of lactose into acids.

The samples from all test tubes with a change of colour of the medium (positive reaction) were subcultured individually to a chromogenic medium: 5-bromo-4-chloro-3-indolyl-β-D-glucuronide tryptone bile agar - BCIG (Oxoid). The plates were incubated for 20-24 h at 44 °C. The blue colonies indicate the action of the β-glucuronidase enzyme, which is the characteristic for most of the *coli* bacteria. The result was interpreted by MPN tables with the number and dilutions of the test tubes in which acid was produced and which were also confirmed as positive by using the BCIG medium, taken in account. The result was given as the number of the *E. coli* bacteria in 100 g of whole flesh.

Results

Aifty-four out of 66 samples examined in 2003 suited the provisions of the Rules (15) concerning marketing. Among the samples that failed to meet the desired criteria, the acceptable number of *E.coli* bacteria was exceeded in all 11 and the coliform bacteria count exceeded in four cases. In one case, the examination only showed a greater number of *E. coli* bacteria. In all 12 samples not suiting the conditions for marketing, the level of 6000 per 100 g was not exceeded; in two of these samples, the value of 6000 was exceeded for the coliform micro-organism count.

Salmonella spp. was present in one sample out of the 116 examined in year 2004. The number of *E. coli* exceeded the maximum limit set in the Rules in 12 samples out of 116 examined in 200;

Table 1: Presence of salmonellas in shellfish samples examined in 2003 and 2004

Year	*Salmonella spp.*/25 g		
	No. of examinations	**not detected**	**detected**
2003	66	66 (100%)	0 (0%)
2004	116	115 (99.1%)	1 (0.9%)
∑	182	181 (99.5%)	1 (0.5%)

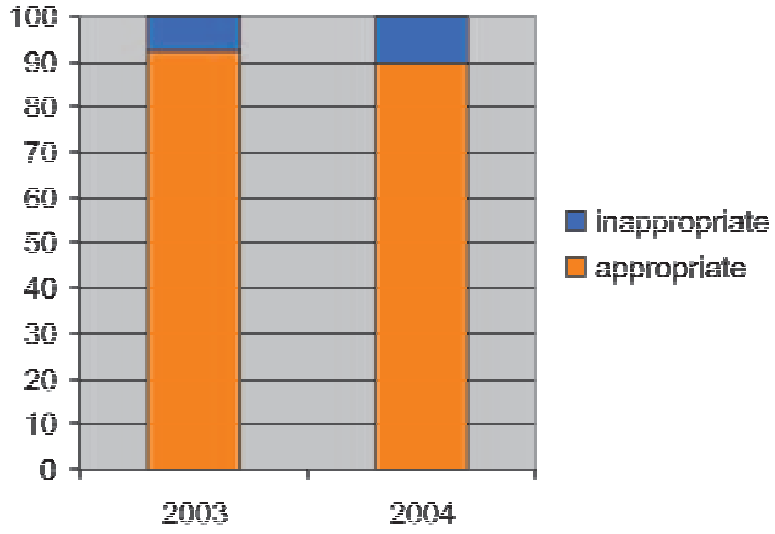

Diagram 1: Percentage of appropriate and inappropriate shellfish samples according to the Rules for direct human consumption of shellfish, regarding demands for *E. coli* number in years 2003 and 2004.

in 11 of these cases the noumber of bacteria present in the sample was between 230 and 4600 and in one sample the number of bacteria was between 4600 and 60000 *E. coli* per 100 g of whole flesh. The other 104 samples examined suited the requirements for the marketing of shellfish.

Discussion

IShellfish feed themselves by filtering sea water and capturing phytoplankton and other nutrients. Through filtration they also consume various contaminants that influence their quality as foodstuffs. Shellfish can contain pathogenic microbes, like salmonellas, *Vibrio parahaemolyticus*, viruses (Hepatitis A virus, *Norovirus*) or parasites (*Giardia*) (4, 9). The main source of shellfish contamination is the faecal pollution of the sea. Faecal contamination of shellfish is related to the organic pollution arriving from inland surface waters, distance of harvest area from the coast, sea temperature and saltines as well as weather – washing of organic substances and micro-organisms deriving from soil into the sea is stronger during heavy rain (14).

The countries along the Mediterranean Sea have reported occasional appearance of salmonellas and the above mentioned viruses in shellfish and shellfish consumption has led to food poisoning in humans (3, 14). In most cases poisoning resulted in mild to moderate gastrointestinal symptoms lasting hours to days, but severe infections like salmonelpsis or hepatitis A occasionally occurred (17).

E. coli and faecal coliforms are not very pathogenic themselves and the purpose of determining their numbers in shellfish is to establish the level of sea pollution. As shown by the parameters set in the Directive, the higher the level of pollution, the greater is the probability of presence of other pathogenic micro-organisms. The procedure is relatively simple, and the result can be obtained as soon as within 48 hours. In this way, one avoids more complicated, time-consuming and expensive determinations of individual pathogenic microbes, like the pathogenic strains of *Vibrio parahaemolyticus*, *Norovirus*, or hepatitis A virus. Shellfish growers want a prompt final result of

examinations, so that they may decide to purify the shellfish in the case of their excessive contamination, and to quickly place the shellfish on the market after purification and re-examination.

The harvesting areas or the sea are classified regarding the content of coliform bacteria or *E. coli* bacteria. The classification is performed by the competent authority after investigating the results of the microbiological examinations of shellfish from a certain area (8). Only shellfish from category A waters, with maximum levels of 230 *E. coli* or 300 coliforms per 100 g flesh, may be marketed for direct human consumption. Shellfish from category B areas must not exceed the limit of 4600 *E. coli* or 6000 coliforms per 100g flesh in 90 % of samples. Such shellfish can only be placed on the market after treatment in a purification station or after relaying so as to meet the category A standards. Live bivalve molluscs from class C areas must not exceed the limits of 60000 fecal coliforms per 100 g of whole flash and should be collected but placed on the market after relaying over a long period (at least two months) in order to meet the category A standards. Where the results of sampling show that the health standards for shellfish are exceeded (more then 60000 faecal coliforms), or that there may be otherwise a risk to human health, the competent authority must close the production area.

The proposal for the new European Directive leaves out the requirement concerning coliform bacteria, but includes the same provision with regard to the number of *E. coli* bacteria (7).

Shellfish we examined were harvested in the Adriatic Sea. In more then 90 % of samples the level of *E. coli* was below limits determined by the Rules. We can't estimate a quality of shellfish on the market, because the shellfish were harvested in different areas and sampled for different purpose (after or before harvesting). We isolated *Salmonella* at 0.5% of examined samples in two years. In Italy, *Salmonella spp*. was detected in 0.7 % of samples analysed between 1996 and 2000 for purposes of microbiological monitoring. Both results reflect a rare but possible presence of these pathogenic bacteria in shellfish from Adriatic Sea (14).

It has been established that bacterial methods do not always reveal the presence of viruses or the presence of members of genus *Vibrio* (13, 10). Depuration is currently commercially practiced and was shown to be adequate for reducing *E. coli*, but ineffective for the elimination of viruses. The shellfish that meet the *E. coli* standards for human consumption may contain human enteric viruses that cause gastroenteritis and hepatitis (11, 6). Hence, there is a need for indicators of viral faecal pollution in order to improve the microbiological control of shellfish.

The EU study (6) discovered that after purification, the level of *E. coli* decrease for 75 %, the level of viruses (by determining the reduction of FRNA – bacteriophage) fell by 43% (28 - 60%) on the average. The reports from Italy show that as much as 50% of the shellfish contaminated by the hepatitis A virus were also positive after purification. Hepatitis A virus was present on average in 10 % of the shellfish tested prior to depuration in Spain and Italy and reduced to 7 % after depuration. It has been also reported that the number of the *Vibrio parahaemolythicus* bacteria may actually increase at temperatures above 20 °C.

All these findings show that current legislative standards for *E. coli* do not effectively protect the consumers from the risk of exposure to pathogenic viruses and other pathogens.

Therefore, in our view, direct determination of *Vibrio* and viruses should be introduced.

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BAKTERIJSKO ONESNAŽENJE ŠKOLJK V SLOVENIJI

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Povzetek: V letih 2003 in 2004 smo pregledali 182 vzorcev školjk, pregledi pa so bili opravljeni v skladu s trenutno veljavnimi predpisi. V vzorcih smo z uporabo metod, ki jih priporoča Evropska unija, ugotavljali pristotnost bakterij vrste Salmonella spp. in število bakterij E. coli. Glede na dovoljeno število E. coli (230 bakterij v 100 g mesa školjk) je veterinarska merila za primernost izdelka za prodajo izpolnjevalo preko 90 % vzorcev. Ta odstotek kaže na majhno verjetnost pojavljanja omenjenih bakterij v školjkah oziroma možnost občasno povečanega števila teh bakterij v školjkah. Pri pripravi novih mikrobioloških meril za živila v Evropski uniji je bilo narejenih veliko raziskav tudi o drugih patogenih dejavnikih, ki lahko povzročajo zastrupitev po zaužitju školjk; mednje sodijo Vibrio parahaemolyticus, Norovirus in virusa hepatitisa A. Znano je, da E. coli in fekalne koliformne bakterije kot bakterijski kazalci onesnaženosti s fekalijami za nadzor okužbe z enteričnimi virusi niso dovolj zanesljivi. Ob tem je pomembno tudi to, da postopki čiščenja oziroma depuracije odstranijo E. Coli in fekalne koliformne bakterije bolj učinkovito kot patogene viruse.

Ključne besede: hrana, onesnaževanje; lupinarji - mikrobiologija; hrana, analize - metode; zakonodaja, hrana