Verotoxigenic Escherichia coli O26 (VTEC) foodborne outbreak in Romania – incriminated foods and test results

INSTITUTE OF HYGIENE AND VETERINARY PUBLIC HEALTH (IHVPH) BUCHAREST

NRL E. COLI ROMANIA

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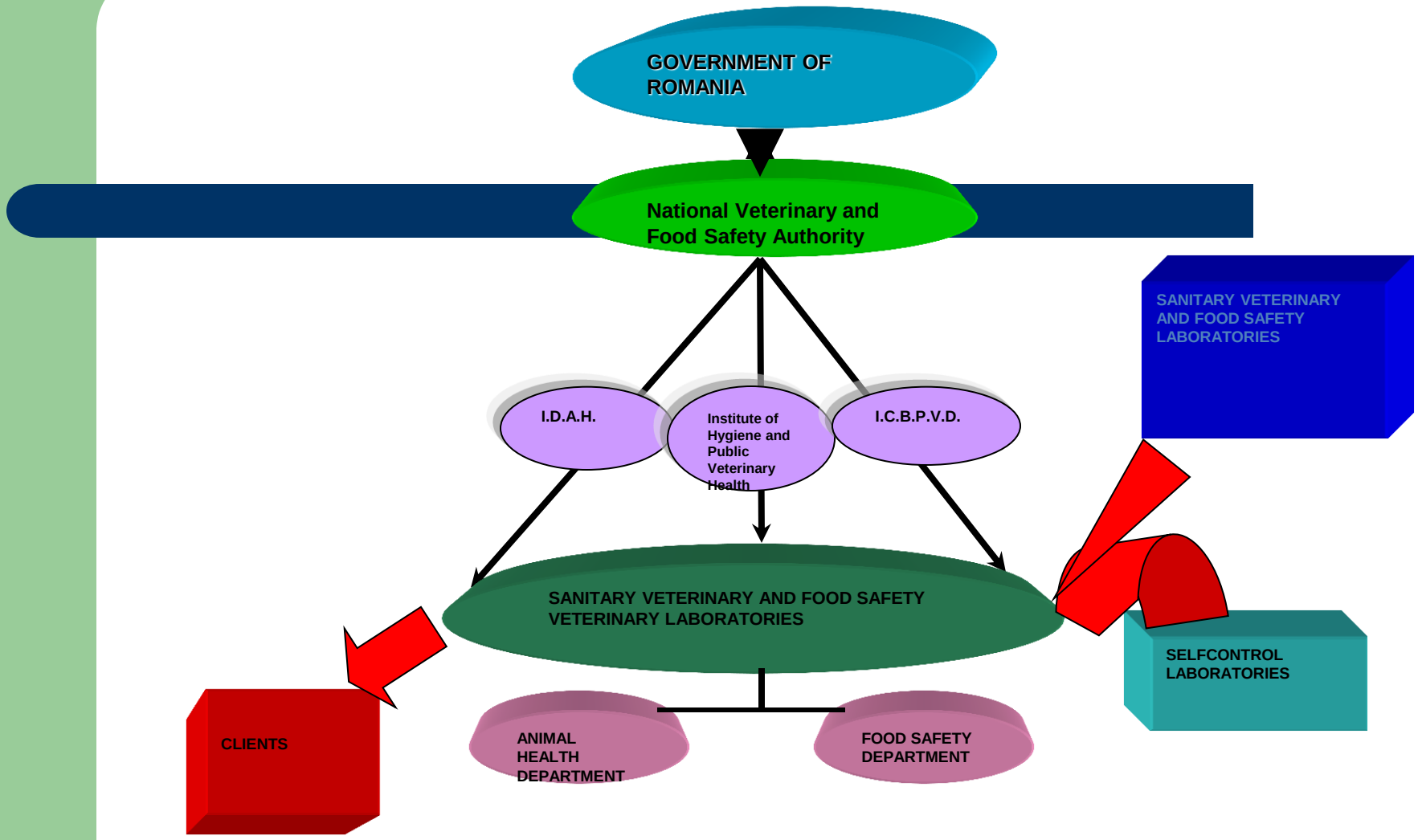
GOVERNMENT OF ROMANIA
National Veterinary and Food Safety Authority
SANITARY VETERINARY AND FOOD SAFETY LABORATORIES
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Preliminary data

- 9 and 10 February 2016: 12 pediatric cases of HUS (initially with diarrhea, some with bloody diarrhea) in children under two years of age from Arges, Bucharest and Dolj counties (25% increasing cases compared to normal). Incriminated STEC genotype: stx2+, eae+, O26.
- 20-26 February 2016: three additional cases were reported in Bacau county (one) and Arges county (two).
- 25 February an investigation team composed by experts from the NIPH - the Romanian Ministry of Health, the European Centre for Disease Prevention and Control (ECDC) presents to NSVFSA a material containing epidemiological investigations collected from patients pointing towards milk dairies, **maybe** a bulk cheese bought from a market in a town from Arges county, as a possible source of infection .

DATA RECEIVED FROM THE EPIDEMIOLOGICAL INVESTIGATION OF THE ROMANIAN MINISTRY OF HEALTH

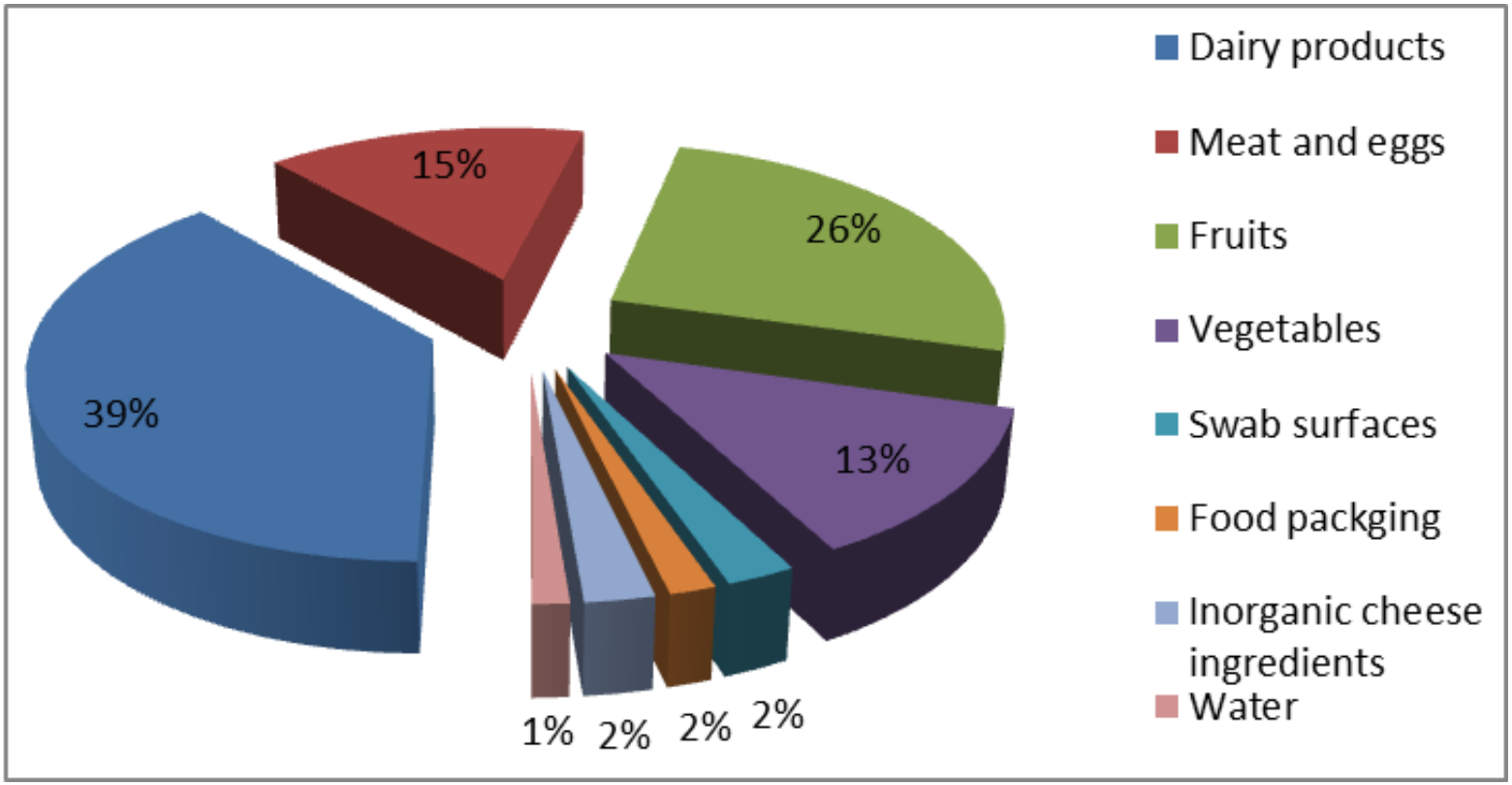
- NSVFSA has started its own investigations on February 25, 2016, following the notification received through the national RASFF from the Ministry of Health which considered certain foods as possible cause for the (HUS) cases; the list of possible incriminated foods, as part of their epidemiological investigation, was updated twice with new cases/foods. The final list contained possible contaminated foods related to 8 children and the data concerning name of the food and the shops from where the products had been purchased/ own household / individuals
- Most of the patients had eaten fruits, vegetables, meat and dairy products, in particular fresh cheese and yoghurt made from pasteurized cow milk
- Fruits and vegetables had been bought either from small local shops or supermarkets with large distribution areas. Vegetables and meat were reported to have been thoroughly cooked before being eaten. Dairy products had been bought from several shops.

FOOD SAMPLING (1)

- **53 different suspect food categories,** sampled by the inspectors from Sanitary Veterinary Directorates at the request of National Sanitary Veterinary and Food Safety Authority, were sent to the laboratory of the Institute of Hygiene and Veterinary Public Health (IHVPH) in Bucharest and tested immediately.

- The food and environmental matrices used in this study were composed of fruits (n=160), vegetables (n=80), swab surfaces (n=15), food packaging (n=10), inorganic cheese ingredients (n=15), water (n=8), chicken meat (n=57), pork (n=10), beef (n=16), eggs (n=2), minced meat (n=10) and dairy products (n=239). Samples taken from five counties were tested according ISO 13136:2012

FOOD SAMPLING (2)



FOOD TESTING – detection method (1)

- **Method steps**: microbial enrichment, nucleic acid extraction, detection of virulence genes (*vtx*1, *vtx*2), *eae* and detection of serogroup-associated genes wzx-O26 and isolation from positive enrichments followed by target genes PCR detection from isolated colonies for confirmation.
- **Buffered peptone water** for supposed VTEC contaminated food matrices like fruits, vegetables, surface swabs, food packaging, food inorganic ingredients, potable water used as an ingredient in food.
- **Modified tryptone soy broth** supplemented with 16 mg/l of the antimicrobial novobiocin for meat and milk dairy products. 25 g or ml of sample were homogenised with 225 ml of enrichment medium and incubated at 37°C for 18 - 24 hours.
- For surface **swab samples**, the enrichment step was done by immersion of the swabs in buffered peptone water followed by incubation at 37°C for 18 – 24 hours. The **fruit and vegetables** were cut in four to five pieces (especially from the surface) that weight 25g and further homogenised in 225 ml of buffered peptone water followed by incubation at 37°C for 18 – 24 hours.

FOOD TESTING – detection method (2)

Dairy products – 2 methods of getting rid of milk fat:

a)1 ml of enrichment → low strength centrifugation (5 min/500xg)→ 900 microliters in new tub → 2 min/15000xg →resuspension in water→ vortex 2 min/15000xg→InstaGeneMatrix extraction.

b) 1 ml of enrichment →2 min/12000xg →discarding the supernatant→addition of 1 ml water→vortex 2 min/12000xg →discarding the water→InstaGeneMatrix extraction (sometimes two or three washing steps were needed especially for butter or milk cream).

200 microliters of InstaGeneMatrix was added to the pellet and the producer standard extraction procedure for bacterial DNA was followed.

Better results: b) – simple, rapid and good spiked validation results.

FOOD TESTING
– detection method (3)

FOOD TESTING – detection method (4)

- The PCR screening step consisted at first by detection of *stx*1 and *stx*2 genes from DNA extraction of 1 ml enrichment broth, followed, in case of a *stx* positive result, by the *eae* and *wzx*-O26 genes detection from the same extract.

- 18 - 24 hours at 37˚C enrichments were dispersed on agar plates with selective mediums like TBX or SMAC/CT-SMAC/RMAC and in the second day fifty colonies were isolated from every sample and pools of ten were obtained followed by *stx* and *eae* PCR genes detection.

FOOD TESTING – detection method (5)

Finally, the intention was to isolate a single bacterial cell that contains the genotype incriminated by the Ministery of Health. So we tested for *stx1/2*, *eae* and the *wzx*- O26.

The amplification of every target gene was performed on Applied Biosystems 7900 HT Fast Real time PCR instrument within a total reaction mixture of 20 µl containing 2 µl of the prepared DNA sample extracted with InstaGeneMatrix® from 1 ml enrichment broth or from colony, 0.5 µM (each) primer, 0.20 µM probe, 10 µl of GoTaq® qPCR Master Mix 2.0 - Promega and PCR grade water to final volume.

FOOD TESTING - detection method (6)

- The real-time PCR conditions consisted of initial denaturation of DNA and Taq polimerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing & extension at 60°C for 1 min.
- Negative control E. coli K12 and EURL positive control strain of STEC O26 (*stx*1+, *stx*2+, *eae*+, *wzx*-O26) were used.

Enormous work volume

Results (1)

- The genes targeted were *stx*1, *stx*2, *eae* and the serogroup associated gene *wzx*-O26, all of them defining the O26 STEC (because this type was the agent stipulated in the outbreak RASFF).
- Between 26 February 2016 and 14 March 2016, in five counties (Argeș, Bacau, Dolj, Ialomita and Vrancea) a total of 574 samples from 141 food items were collected from 24 different premises. The results showed that from 16 types of food (five dairy products, ten poultry and one mixed meat (beef and pork) collected in all the five districts, 86 samples were positive by real-time PCR for one or more of the investigated genes (*stx*1, *stx*2, *eae*, *wzx*-O26) as displayed in Table 1.

Results (2)

Table 1 PCR and isolation findings in the analyzed food samples

Nr. crt.	*Matrixes*	*Number of samples*	*Characteristics of positive findings*
1.	Cheese from cow's milk	5	PCR: eae+O26+ Isolation: E. coli stx2-,eae-,O26+ present/25g
2.	Cheese from cow's milk	25	PCR: stx2+ eae+O26+ Isolation: E. coli stx2-,eae-,O26+ present/25g)
3.	Chicken meat	21	PCR: eae+O26+ Isolation: E. coli eae+,O26+ absent/25g)
4.	Chicken meat	25	PCR: eae+O26+ Isolation: E. coli eae+,O26+ present/25g)
5.	Mix beef and pork meat	5	PCR: stx1+ stx2+ Isolation: E. coli O26 absent/25g)
6.	Cow's milk	5	PCR: eae+O26+ Isolation: E. coli eae+,O26+ absent/25ml)
Total		86	

Results (3)

- We detected the target genes of STEC O26 by PCR but unfortunately without STEC O26 isolation, at first, in a batch of bulk soft cheese in a store from a town included the epidemiological study of the team CDC – Ministry of Health. Immediately, veterinary inspectors from Arges county went to the manufacturer premises to check - the quantity of cheese batch was 25 kg produced on 22/02/2016, which amount has not been recovered to the manufacturer or marketing.

- Afterwards, another sample brought from another store, by different inspectors, but from the same manufacturer and the same batch of bulk soft cheese, was tested. The result were positive again but only for PCR.

Results (4)

Measures taken by NSVFSA:

- Cessation of activities in the cheese department of the plant (where the fresh cheese was produced), until clarification of the situation
- Employees examinations – stools were tested in the Cantacuzino Institute with positive results
- Sampling of raw milk from both farms at the reception for bacteriological examination and sampling of water from the entry and exit of the unit
- The incriminated FBO announced that voluntary closed the activity in all production departments

Results (5)

The Romanian FBO has suspended work on its own initiative, on February 28, 2016.

Of the 574 samples, 45 from 9 different dairy products were collected from the dairy plant belonging to the Romanian FBO and analyzed for STEC. Five samples from a single dairy product type (cheese from cow's milk) were found presumptively positive for STEC O26 highly pathogen (the enrichments were positive for *stx*2 gene). However, in this sample were isolated strains of *E. coli* O26 but without verotoxigen genes *stx*2.

Stool tests from 19 employees: 7 were found positive for *E.coli* and, from those, 3 were found positive for *stx* and *eae* genes of VTEC) (Cantacuzino Institute results).

Results (6)

- Moreover, 25 of the 574 samples of cheese from cow's milk from five different dairy products collected from other premises than the Romanian operator's, but produced by the Romanian operator, were found presumptively positive for STEC O26: 15 samples of three batches of dairy product collected at a local supermarket in Arges county and 10 samples from two batches of different dairy products collected in two local hospitals, both in Arges district.
- An additional 44 non-food samples were collected at the premises of the Romanian operator: drinking water, environmental samples, and cheese ingredients (rennet, calcium chloride and salt). Environmental samples were taken from the surface of various pieces of working equipment and components of the processing machinery, as well as from cheese packaging material. All of them were found negative.

Results (7)

- Related to the Romanian FBO which produced the presumptive positive cheese, two farm supplying cow milk were investigated for the presence of STEC in raw milk. At first a sample of the raw milk was presumptive positive only to *eae* and *wzx*-O26 to PCR, but without isolation. Another sample taken afterwards was negative. Four additional water samples (mains, water tank, pond and waste water from milking equipment) and one bovine fecal sample were negative for STEC. Raw milk samples collected at the second farm were negative for STEC.

Results (8)

- Chicken meat and mixed meat (beef and pork), detected positive for *eae* and *wzx*-O26 were sampled because these foods were less or more related to another STEC cases appeared later in March in Bacau and Ialomita districts. But, there was no detection of verotoxin encoded genes in the chicken meat enrichments, so there was no presumptive presence of the STEC in these samples.
- All fruits and vegetables samples were PCR negative for the target genes *stx*1, *stx*2, *eae* and *wzx*-O26.
- For all presumptive positive cheese samples, the Ct's in the real-time PCR were higher than 35 for *stx2* and *eae* genes, as for *wzx*-O26 the Ct's were about 21 to 23.

Results (9)

Isolated colonies from cheeses (*stx*1-, *stx*2-, *eae*-, *wxz*-O26+) and chicken meat samples (*stx*1-, *stx*2-, *eae*+, *wxz*-O26+) were submitted to Cantacuzino Institute from Bucharest, were these have been compared to the children's isolates by PFGE. The PFGE profiles of the human isolates differ from the cheese (in fact, these were strains recovered from different fragments of the same batch) and chicken isolates (also recovered from different fragments of the same batch product) from March 2016, so we can not conclude that the outbreak is due to a single source.

Conclusions (1)

- Of all foods analyzed during the outbreak, only cheeses from one producer (incriminated also after performing epidemiological investigation) were presumptive positive to at least one of the target virulent genes and have been linked to the outbreak as related human health authorities.
- In such a food-borne outbreak of VTEC, even if the tests must be performed in accordance with ISO 13136:2012, it’s good to follow the detection of all genes involved in the disease because during infection it is possible to lose the incriminated genes. Any information relating the target genes involved is required in establishing epidemiological outbreak sources.

Conclusions (2)

- Ct's values greater than 25 may not lead to an sure achievement regarding isolation of bacterial cells. Taking into account that the isolation of a VTEC strain may also depend on the competition with background microflora, in some cases we could have low concentrations of VTEC but also low background microflora, giving the possibility to isolate the targeted bacteria.
- Even if the chicken meat has been incriminated as a potential source of the outbreak, in their enrichment broths we did not found any of the verotoxin encoding genes.

Conclusions (3)

- For some cases, person to person contamination could it be possible from parents / subclinical carriers to their children.
- The NSVFSA included this year (and it is undergoing) a monitoring program for VTEC from swabs carcasses, cow, sheep, goat meat/milk and meat/milk products over the entire country.

Conclusions (4)

This outbreak lay out the need of the cooperation between physicians and vets in combating foodborne diseases, highlighting the Louis Pasteur words:

"*A human doctor saves the individual, a veterinarian saves mankind*".

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OUR GOAL

Thank you !