Antibody capture ELISA for the sensitive direct detection of heat-stable toxin of Escherichia coli from soft cheese

G. Hahn, B. Bonniksen

Institute for Hygiene, Federal Dairy Research Centre, Kiel

1 Introduction

Within the genus Escherichia coli (E.coli) during the last decades different mechanisms of pathogenicity could be detected (EPEC, EIEC, ETEC, VTEC/EHEC). On the background of the EU Milk Hygiene Directive 92/46 in certain milk products the presence of pathogenic E.coli strains has to be investigated if a maximum count (M) is exceeded. For the direct isolation and identification of heat-stable toxin (ST) of enterotoxigenic E.coli (ETEC) from soft cheese a method should be developed. Normally, in food no toxins are produced although toxigenic strains are present and multiplying. To save time and material the laborious isolation of respective E.coli colonies should be avoided to get quicker results regarding positive products. The idea was to incubate the untreated sample in special media to enhance the production of toxins and to detect these by an antibody capture ELISA.

2 Material and methods

2.1 Immunoassay

As an antigen for immunization E.coli heat-stable enterotoxin Sta (SIGMA E 5763) was used and transformed to an immunogene by a modified method according to AVRAMEAS (1) by linkage to glutaraldehyde.

Immunization of rabbits was done every ten days starting with 70 µl of the immunogen and continuing with 140 µl, 280 µl and 560 µl. The immunogene was coupled to Alugel S (Serva) and Freund adiuvans. A booster shot was set after 4 and 12 weeks after last immunization. The determination of the antibody titer and the performance of the ELISA was described previously (4).

2.2 Sample preparation for toxin detection

Sample material: Three soft cheeses with different contamination flora from the retail.

Inoculation of 10 g cheese in 90 ml prewarmed EE broth (Oxoid BM 317) with a ST-producer in different bacterial counts (10^3, 10^2, 10^1/g cheese).

Homogenization two minutes in the stomacher

Incubation in a shaking water bath, 4h at 37° C

Subculturing of 4 µl of the filtrate in 4 ml Caye 2-broth

Incubation 18h at 37° C in a shaking water bath

Spin down of the bacteria, 15 min at 3000 g

Dilution of the supernatant 1:5 in Caye 2-broth for the prevention of unspecific reactions

Detection by ELISA

3 Results

3.1 Immunoassay

Principle of evaluation

The extinction values are given as relative optical density (%OD). These are calculated by setting the zero values (control) as 100 % because it is a competitive ELISA. The meaning of %OD is that the extinction values of more or less positive samples are brought into percentage relation to 100 % (control). By this principle accidental differences from day to day are levelled out. In Figure 1 the following results are given:

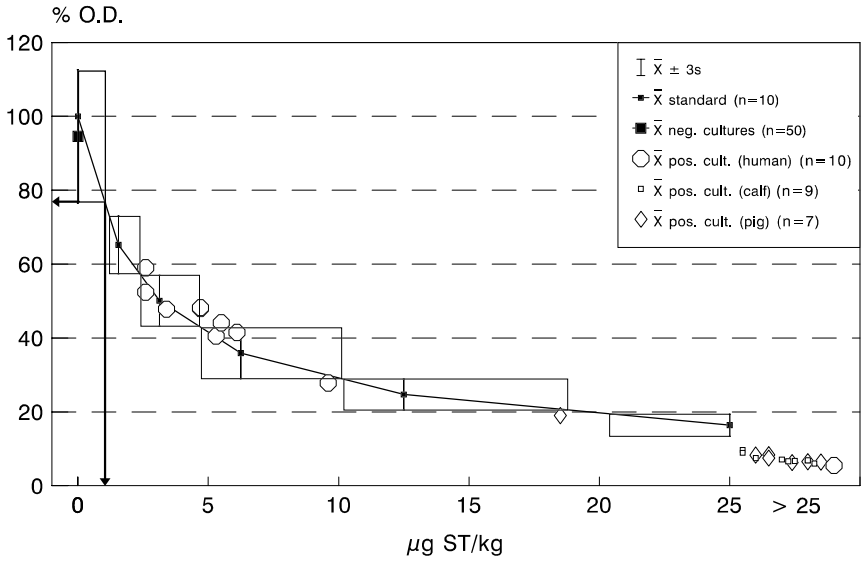

Figure 1: Standard graph of an E.coli ST-ELISA including readings of ST-negative and ST-positive reference strains

Pure toxin in dilution steps from 0 to 25 ng/ml was measured ten times independently. Considering a threefold standard deviation the results are shown in the graph. The height of the boxes indicate the threefold standard deviation at a given concentration. As one sees they do not touch one another which enables a quantitative identification. Additionally, the values allow the determination of the threshold (± 3s) between positive and negative samples expressed as %OD.

The obtained values of 15 different ST-negative strains from 3-5 repetitions showed a mean of 94.6 %OD compared with the 100 %OD from toxin free controls. By subtraction this resulted in a sharp threshold between positive and negative samples at 76.6 %OD regarding ± 3s. (see horizontal arrow).

Readings of positive reference strains (26 values from 12 strains of 1-5 tests) are indicated by different symbols regarding the origin from humans, calves and pigs. It is to be seen, that the strain from humans show a distinct lower toxin production than those of bovine and porcine strains.

The detection threshold (sensitivity) is to be estimated at about 1.0 µg ST/kg if you throw a line from the point of intersection of the threefold deviations of the zero-values and the positive readings (see vertical arrow).

The titer of antiserum is between 1:10,000 and 12,000.

The specificity of the test was checked by two independent tests using as substrates choleratoxin, all known S.aureus enterotoxins; all readings were negative. Out of 15 verotoxin (Shigatoxin) producing strains 13 were negative and two positive, obviously producing both toxins.

The repeatability is indicated by the coefficient of variation (cv) of the standard readings with pure toxin (n = 10 per concentration) of 5.36 and the ST negative cultures (n = 50) of 6.34.

Although for routine analysis it is sufficient to have a clear cut between positive and negative samples ST dilutions from 0-25 ng/ml were tested in a blind study by three independent repetitions: 57 of 60 samples could be determined within the respective $\pm$ 3s section quantitatively.

According to a routine test three isolates each of 70 French raw milk soft cheeses were examined and showed to be clearly negative.

3.2 Toxin identification in cheese

By artificially inoculated soft cheeses even 10 cfu of ST producing E.coli per gram could be identified in spite of a high contamination flora of coliforms and ST negative *E.coli* (Table 1).

The low amount (4 µl) of culture from EE broth to be subcultered in Caye 2-broth resulted from separate tests. Using e.g. 0.33 and 0.16 ml the test was not to read, obviously, because of too high amounts of sample matrix.

Table 1: Identification of heat-stable toxins (ST) free soft cheese with different levels of contamination flora after artificially inoculation with ST producing E.coli

Contamination flora/g		ETEC (ST)	ST
Coliforms	E.coli	g/cheese	ng/ml
4.3×10^4	2.4×10^3	6.5×10^2	> 25
		7.6×10^1	1.7
		7.3×10^0	0
2.4×10^5	3.8×10^1	7.6×10^2	> 25
		7.9×10^1	> 25
		7.6×10^0	3.1
4.3×10^4	1.5×10^2	1.4×10^3	> 25
		1.5×10^2	> 25
		1.1×10^1	9,0

4 Discussion

Similar immunoassays described in the literature (2, 5-9, 10, 11) are mainly based on selfmade antigens by complicated procedures. In contrary to this we used a commercially available toxin for immunization and coating of the solid phase for the ELISA. The necessity of a simple test results from the EU Directive 92/46 where the identification of toxigenic E.coli is asked. The advantage of the test described is that those strains may be identified also in heavily contaminated samples. Because, obviously, in spite of the presence of toxigenic strains no toxin is produced in the substrate, normally you have to isolate at random suspicious colonies by laborious enrichment methods. This problem was avoided by stomaching and incubation of the sample in a selective medium (EE broth) to enhance the growth of E.coli and subculturing in Caye 2-broth for optimum toxin production. The toxigenic strains present in the sample thereby can be detected directly by

their toxin. Even seven cfu/g of soft cheese could be detected in spite of a high contamination flora. These principle has proved yet for heat labile toxin of ETEC strains (3). The advantage without any doubt is a saving of time and material and offers a more reliable test than the at random isolation of e.g. 5 colonies from a sample from a certain dilution step. This principle may be transformed to other toxigenic bacteria species in food hygiene.

5 Summary

On the background of the EU Milk Hygiene Directive 92/46 a simple, sensitive and specific ELISA for the detection of heat-stable Escherichia coli toxin (ETEC-ST) was developed. The principle is a competitive antibody capture test. For immunizing rabbits a commercially available toxin (Sta, SIGMA) was used. The test allows to detect within a few hours the toxin from a culture supernatant in a sensitivity of 1 ng/ml - qualitatively and quantitatively. The at random isolation and further handling of suspicious colonies from a selective medium normally is very laborious. Therefore, an additional method was developed by incubating the sample, e.g. 10 g of cheese in the selective EE-broth which is subcultured and incubated again in Caye 2-broth for enhancing the toxin production where from the toxin can be identified. Even at a contamination flora of more than 10^5 coliforms/g and 10^1 E.coli yet 7 toxin producing E.coli/g in the original flora could be detected. This method enables a large saving of material and time and, additionally, is more reliable to detect toxin producers in a sample than the at random isolation of suspicious colonies.

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