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Detection of antibiotics in goats' milk: comparison of different commercial microbial inhibitor tests developed for the testing of cows' milk

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ABSTRACT

The aim of this study was to evaluate the impact of the composition and the quality of goats' milk on the rate of false positive results obtained for microbial inhibitor tests developed and validated for the testing of cows' milk. Milk samples of 200 different individual goats were tested by nine microbial inhibitor tests: BR-AS Special, CMT-Copan Milk Test, Delvotest SP-NT, Delvotest T, Brilliant Black Reduction Test MRL, Charm Blue Yellow II, Charm CowSide II, Eclipse 100 and Eclipse 3G. Each test was performed *in duplo*, and the interpretation of the results was carried out visually and instrumentally. Samples initially tested as positive were retested and also tested after a milk pre-treatment (heat treatment, fat removal, fat removal followed by heat treatment). The results showed that most of microbial tests commonly used for bovine are suitable to test goats' milk giving a rate of false positive results < 5 %, except for BR-AS Special, Charm Blue Yellow II and Delvotest SP-NT. The visual interpretation of the results decreased the test specificity. The most appropriate milk pre-treatment to reduce the number of positive results for almost all tests employed was fat removal followed by heat treatment.

Keywords: antibiotics, natural inhibitors, goats' milk, microbial inhibitor tests, false positive results.

1. INTRODUCTION

Currently, antibiotic residues in milk are still of great concern to different sectors such as milk producers, the dairy industry, regulatory agencies and consumers. As milk production by small ruminants increased in recent years, the use of antibiotics in dairy goats has become a usual practice in veterinary medicine to treat mastitis and other diseases (Buswell, Knight, & Barber, 1989; Silanikove, Leitner, Merin, & Prosser, 2010).

The problem is that many antibiotics used in dairy goats are specially registered for dairy cattle and the withdrawal period cannot be extrapolated accurately, since the depletion data were generated for a different animal species (Ferrini, Trenta, Mannoni, Rosati, & Coni, 2010; Karzis, Donkin, & Petzer, 2007). Therefore, antimicrobial residues could still be present in goats' milk after respecting the withdrawal time set for cattle (Petzer et al., 2008). Veterinary drug residues in milk might pose a risk to health, generating allergic or toxic reactions (Alanis, 2005; Demoly & Romano, 2005; Sanders, Bousquet-Melou, Chauvin, & Toutain, 2011) and technological implications in the manufacturing of dairy products (Adetunji, 2011; Berruga, Beltrán, Novés, Molina, & Molina, 2011; Packham, Broome, Limsowtin, & Roginski, 2001). The problem is even bigger for goats' milk since it is mainly intended for the production of cheese or yogurt.

The European Union established Maximum Residue Limits (MRLs) for veterinary medicinal products in Commission Regulation (EU) No 37/2010, as foreseen in Commission Regulation (EU) No 470/2009. Inhibitory substances in milk are routinely screened at farms, dairies and laboratories. Currently, several commercial methods to detect antibiotics are available (IDF, 2010). Microbial inhibitor tests are the most used, because they are quick, easy to use, and relatively cheap and can detect a wide spectrum of compounds (Comunian, Paba, Dupre,

Daga, & Scintu, 2010), they are generally based on the inhibition of the growth of the microorganism *Geobacillus stearothermophilus* var. *calidolactis*, and their results are qualitatively interpreted by a colour change (yellow: negative and blue/purple: positive). Evaluating the performance of screening tests, requirements are stipulated for the rate of false compliant results. Following Commission Decision 2002/657/EC this rate should be < 5 % (β-error) at the level of interest (CCβ). In the same Commission Decision, as a general requirement for specificity it is stated that a method should be able to distinguish between the analyte (antibiotic residue) and the other substances under the experimental conditions. Therefore, specificity is associated with the presence of false positive results and is of great interest to evaluate the analytical capacity of a test. But the legislation is not fixing levels for the rate of false positive results. A positive test result is considered to be false positive when no antibiotics are present in the milk. To determine false positive results, a large number of milk samples from animals not treated with veterinary medicinal products should be analysed.

Microbial inhibitor tests are not specific for just antibiotic residues but may be affected by any substance or compound capable of inhibiting the growth of the test organism. Several factors could contribute to false positive results such us natural inhibitors (lactoferrin, lysozyme) (Carlsson, Björck, & Persson, 1989), a high somatic cell count (Andrew, 2001), an abnormal fat content (Reybroeck & Ooghe, 2012), detergents and disinfectants (Salomskiene, Macioniene, Zvirdauskiene, & Jonkuviene, 2013; Zvirdauskiene & Salomskiene, 2007), and preservatives (Molina, Segura, Luján, Althaus, & Peris Ribera, 2003).Inhibitor tests have been developed for the testing of cows' milk, but are also used for the analysis of milk from other species, such as goats. Most of studies about non-compliant results were performed with cows' or ewes' milk (Althaus et al., 2003; Andrew, Frobish, Paape, & Maturin, 1997; Beltrán, Berruga, Molina, Althaus, & Molina, 2015; Kang, Jin, &

Kondo, 2005; Molina *et al*., 2003). No to limited information for goats' milk is available despite the fact that goats' milk, due to its more extreme composition, is likely to cause a higher rate of false positive inhibitor test results compared to cows' milk. Some kit manufacturers do not specify the animal species when talking about milk, in other kit inserts the suitability to test goats' milk is specifically mentioned. In general very limited information is given about the possibility to obtain false positive results due to natural inhibitory substances or other interferences.

False positive results can have serious consequences, as producers and the dairy industry are encountered with economic losses. Good milk will be discarded and a financial penalty will be given as a legal consequence for a positive test result in regulatory testing of milk. Validation of the tests for goats' milk is very important for the selection of the most appropriate testing strategy for a correct interpretation of the test results and to ensure good monitoring of antibiotics in dairy goats' milk.

Thus, the aim of the study was to compare the response of most microbial screening methods developed for cows' milk, which are currently used for the monitoring for antimicrobial residues in goats' milk. A second aim was to try to limit the rate of false positive results by the application of a milk pre-treatment as heat treatment, fat removal, fat removal followed by heat treatment.

2. MATERIAL AND METHODS

The experimental study was carried out in the Technology and Food Science unit (Melle, Belgium) of the Institute for Agricultural and Fisheries Research (ILVO-T&V).

2.1. *Milk samples*

Two hundred individual milk samples of different goats of White Saanen breed were collected from three Flemish goats' farms with different feed management (ecological: Johan Van Waes, Lochristi-Zaffelare and 't Eikenhof, Lokeren; and conventional: 't Leenhof, Zele).

The sampling of milk of different individual goats was performed in the afternoon milking, around 5 and 6 p.m on all farms. Each sample consisted of some 600 mL of individual goats' milk and was kept refrigerated at ≤ 4 °C until transport to the laboratory the next morning. On arrival, the samples were homogenized and divided in several aliquots (50 mL) to perform the screening tests for antibiotics (first and second day), the remaining milk was frozen at -30 °C in different flasks and volumes for additional residue analysis.

2.2. Microbial inhibitor tests

Milk samples were tested 14-20 hours post-milking by means of nine different microbial inhibitor tests: BR-AS Special, CMT-Copan Milk Test, Delvotest SP-NT and Delvotest T from DSM Food Specialties (Delft, The Netherlands), Brilliant Black Reduction Test MRL (BRT MRL) from Analytik in Milch Produktions- und Vertriebs-GmbH (Munich, Germany), Charm Blue Yellow II and Charm CowSide II from Charm Sciences Inc. (Lawrence, MA), Eclipse 100 and Eclipse 3G from ZEULAB S.L. (Zaragoza, Spain). All tests are based on the inhibition of the growth of the microorganism *Geobacillus stearothermophilus* var. *calidolactis*. The color indicator in most of the methods used is bromocresol purple, but for the BRT MRL and BR-AS Special it is brilliant black. Of all kits, the 96-well microtiter plate format was used, except for Charm CowSide II which was in individual test vials. The commercial tests were stored between 4 and 8 °C and used following the instructions of the kit manufacturers. In every run of each inhibitor test, blank reference milk (mixture of 6 negative goats' milk samples) and antibiotic standards were included, these last doped in blank goats' milk at different concentrations depending on the detection capabilities of each method. Oxytetracycline (O5875), benzylpenicillin (PENNA), sulfadiazine (S8626), and sulfadoxine (S7821), all from Sigma-Aldrich (Bornem, Belgium) were used as control standards. The milk volume added to the wells and the test vials was 100 μL in all methods

except in Charm Blue Yellow II it was 50 µL. For all milk samples each microbial inhibitor test was performed in duplicate.

All microbial tests were incubated in a covered waterbath (Type 19 + MP thermostat from Julabo Labor-technic GmbH (Seelbach, Germany)) at 64.0 ± 0.2 °C, except for the Charm CowSide II test vials that were incubated in a Charm digital dry block incubator 220V (Charm Sciences Inc.), Eclipse 100 and Eclipse 3G plates were incubated in a FX incubator (ZEULAB S.L) at 65 °C. The incubation time is different between the microbial methods employed, the BR-AS Special has the shortest incubation time (2 hours), whereas the Delvotest T, Charm CowSide II and Charm Blue Yellow II present the longest (± 3 hours), other methods have intermediate times. In microbial inhibitors tests this length of incubation is set by the manufacturer or indicated for the specific batches. However, some microbial tests as BRT MRL, Charm CowSide II, Charm Blue Yellow II, Delvotest SP-NT and Eclipse 3G required a longer incubation time (10 to 25 min) to obtain negative results for the reference blank milk controls on each plate possibly because the indicated incubation times are set for cows' milk..

The interpretation of the results was carried out visually and instrumentally, except for the Charm CowSide II test which was interpreted only visually. The instrumental interpretation for BR-AS Special, Delvotest SP-NT and Delvotest T plates was done by means of a flatbed scanner (HP Scanjet 7400C, Hewlett-Packard Company, Palo Alto, CA) connected to DelvoScan software, version 3.05 (DSM Food Specialties); the cut-off was set at a Z-value = -3.00. For CMT plates a HP GRLYB-0307 flatbed scanner (Hewlett-Packard Company) connected to CScan software, version 1.32 (Copan Italia S.p.A., Brescia, IT) was used; the cut-off was set at a CIF value = 4.5. Charm Blue Yellow II results were interpreted by Epson Perfection V30 (Epson America Inc., Long Beach, CA) flatbed scanner and GVSCAN software version 1.1 (GEVIS, Fidenza, IT); the cut-off was set at a SCORE = 6.00. BRT

MRL, Eclipse 100 and Eclipse 3G results were interpreted photometrically using a spectrophotometer (Multiskan EX, Thermo Scientific, Waltham, MA) with 450 nm (filter 1) and 620 nm (filter 2) for BRT MRL; the cut-off was fixed at a threshold value=40%, as recommended by the commercial company. The threshold value (%) was calculated for each plate by measuring the absorbance of eight negative and positive controls (NC and PC, respectively), using the following conversion formula: (average sample absorbance - average NC)/(average PC- average NC) × 100 = % value. Eclipse 100 and Eclipse 3G were read using 590 nm (filter 1) and 650 nm (filter 2), the cut-off were set by the average absorbance for eight blank goats' milk samples increased by 0.3 or 0.2, respectively. By visual interpretation the samples were evaluated as "negative" (yellow color), "positive" (blue-purple color), and doubtful (intermediate colors between yellow/blue-purple).

2.3. Treatments of positive milk samples

To check that all milk samples used in the study were free of antibiotic residues, the positive milk samples for any microbial inhibitor test were tested the day after with the addition of β-Lactamase ES (Sekisui Enzymes West Malling, UK), 4-aminobenzoic acid (PABA) (Sigma-Aldrich, Bornem, Belgium) or CaCl2 (Merck KGaA, Darmstadt, Germany) and by means of different group-specific receptor-binding assays (Twinsensor BT, 3SENSOR, and 4SENSOR from Unisensor s.a. (Liège, Belgium); Charm MRL BLTET2 from Charm Sciences Inc. (Lawrence, MA) and βeta-star from Neogen Corporation (Lansing, MI). After the analyses by rapid tests, the positive samples were confirmed with a chromatography method (LC-MS/MS) at ILVO as described by Daeseleire, De Ruyck, & Van Renterghem (2000).

Also, milk samples testing positive in the initial residue screening, were retested after the different milk pre-treatments to try to reduce the number of false-positive results for goats' milk and hence establishing the best strategy for analysis by each microbial method. Following sample treatments were tested: heat treatment (80 °C for 10 min), fat removal

(centrifuging at 3,100 g for 10 min at 4 °C, then removal of the fat on the top with cotton tipped applicators, and the last treatment was fat removal followed by heat-treatment). Besides, milk without any treatment was analyzed.

2.4. Statistical Analysis

The differences between the reading system used for the interpretation of the microbial tests results (visual and instrumental) were tested with McNemar's test. Statistical analyses were performed using SAS, version 9.2, 2001 (SAS Institute Inc., Cary, NC).

3. RESULTS AND DISCUSSION

The Table 1 shows the specificity (false positive rate) by the visual and instrumental reading of different commercial inhibitor tests developed for cows' milk. According to the instrumental interpretation, the Copan Milk Test, Eclipse 100 and Delvotest T presented a high specificity (99-99.5 %), obtaining a false positive rate between 0.5 and 1 %. The Charm CowSide II (73.4 %, visual reading) and the BR-AS Special (77.9 %) presented a much lower specificity (77.9 %) compared to the other microbial inhibitor tests studied. Charm Blue Yellow II (94.5 %) and Delvotest SP-NT (93.9 %) and were also showing a specificity < 95 % (> 5% of false positive results). It is worth noting that following the kit inserts the Delvotest T, Eclipse 100 and Eclipse 3G are suitable to be used for the testing of goats' milk, while for BR-AS Special, Brilliant Black Reduction Test MRL (BRT MRL), Charm CowSide II, CMT-Copan Milk Test and Delvotest SP-NT just 'milk' without any specification is indicated as matrix and for the Charm Blue Yellow II specifically 'cows' milk'.

For some tests, a significant agreement was found between visual reading and instrumental reading (Copan Milk Test, Eclipse 3G, Delvotest T, and Delvotest SP-NT MCS), whereas for the other tests, no significant agreement was found (BR-AS Special, BRT MRL, Eclipse 100, and Charm Blue Yellow II). In all latter tests, the specificity calculated by visual reading was lower compared to instrumental interpretation (Table 1).

These differences indicated more positive results and hence faster penalization for a visual reading of the test results for goats' milk (BR-AS Special, BRT MRL, Eclipse 100, and Charm Blue Yellow II). It is important to mention that with each microbial inhibitor test for goats' milk intermediate colors (green-yellow, yellow-blue) were obtained which most of the time were classified visually as positive or doubtful, and instrumentally close to the cut-off established for cows' milk by the kit and software manufactures. Testing cows' milk, Stead et al. (2008) observed that the visual and scanner reading for Delvotest SP-NT in ampoules and multi-plate format gave comparable results. However, these authors also indicated that the visual assessment of samples with intermediate colours (purple in a yellow background) is more difficult and such colours are often interpreted as a suspect positive result.

Some authors (Kang & Kongo, 2001; Molina, Segura, Luján, Althaus, Peris Ribera, 1999, Zaadhof, Schulze, & Maertlbauer, 2004) indicated that the number of false positive results is influenced by the incubation time, a longer incubation period produces less positive or doubtful results in cows', ewes' and goats' milk.

Despite the fact that the specificity with instrumental reading is more convenient, the visual interpretation of results in microbial inhibitor tests is prevalently used in farms (ampoule versions), dairies and laboratories which may not have the equipment and software to perform the instrumental reading.

The false positive rate (visual interpretation of the results) indicated by Beltrán, Berruga, Molina, Althaus, & Molina (2015) who tested individual goats' milk from Murciano-Granadina breed with microbial inhibitor tests was similar (0.6-4.3 %) compared to the values found in this study for instrumental reading (0.5-6.1 %), except for BR-AS Special (22.1 %) but lower for visual interpretation in most of the tests (6.1-34.2 %). It is important to mention that milk samples from individual animals, which present a higher variability on composition and quality parameters, while in control quality programmes in general bulk milk samples are

analysed, which present a lower percentage of false positive results (Comunian, Paba, Dupre, Daga, & Scintu, 2010).

On the other hand, no false negative results were found, because one of the positive samples was confirmed by chromatographic analysis (LC-MS/MS) for tetracyclines (< 10 µg/kg) at ILVO laboratories, and all microbial inhibitor tests detected it. Therefore, this sample was removed from the study (n= 199).

To try to deepen and better understand the differences between visual and instrumental readings of the results from microbial inhibitor tests, the samples were classified into 4 categories (1, 2, 3 and 4) based for instrumental reading on following cut-off values calculated for each test: cut-off - 3×SD (standard deviation), cut-off, and cut-off + 3×SD and the four classes for visual reading were based on the colour of the test medium after incubation: yellow, intermediate yellow-blue, intermediate blue-yellow, and blue-purple (Table 2). A large percentage of milk samples analysed in this study (Table 1), presented questionable and/or positive results for visual readings, which were classified in categories 2, 3 and 4 in Table 2. This fact indicates that the values close to the cut-off may have intermediate coloration that hinder their interpretation. It should also be noted that in many cases the final result of these milk samples which are close to the cut-off might depend on the negative controls used to verify the correct operation of the plates since the incubation time can become too long or too short depending on the nature and composition thereof.

To try to reduce the number of false positive results obtained for microbial inhibitor tests all milk samples testing positive (instrumental reading) in the initial residue screening, were retested the next day (Table 3) without any treatment and after the application of three different milk pre-treatments (heat treatment, fat removal and fat removal followed by heat-treatment). The retesting of positive samples after one day without any milk pre-treatment in some tests reduced the number of positive outcomes on microbial inhibitor tests, especially for

Eclipse 3G and Charm Blue Yellow II, with a decrease from 4 to 2 and 11 to 5 positive samples, respectively (Table 3).

The microbial inhibitor tests BRT MRL and Delvotest T were not influenced by the milk pre-treatment. However, the best milk pre-treatment on most microbial tests was the fat removal followed by heat treatment reducing practically in all cases all positive results. For the BR-AS Special the most effective milk pre-treatments were fat removal and fat removal followed by heat treatment reducing the number of positive outcomes from 44 to 10 and from 44 to 9, respectively .

With the implementation of fat removal followed by heat-treatment as milk pre-treatment the specificity of all microbial tests for testing for antimicrobials in goats' milk was > 95 % (BRT MRL: 97.5 %, CMT-Copan Milk Test: 100 %, Charm CowSide II: 100 %; Eclipse 100: 100 %; Eclipse 3G: 100 %; Charm Blue Yellow II: 100 %; Delvotest T: 99.5 %, and Delvotest SP-NT MCS: 98 %), except for BR-AS Special (82.4 %).

Some authors who tested cows', ewes' or goats' milk have suggested the use of heat treatment to diminish the occurrence of false positive results in microbial inhibitor tests, although they used different heating temperatures and times (82 ºC for 5 min (Kang & Kondo, 2001; Oliver, Duby, Prange, & Tritschler, 1984) and 82 ºC for 10 min (Molina, Segura, Luján, Althaus, Peris Ribera, 1999; Molina et al., 2003)).

Despite the beneficial effect of the heat treatment on the specificity of microbiological screening tests, one should take into account the possible degradation or antimicrobial activity losses of the antibiotic thermolabile substances eventually presents in milk due to the high temperature. In fact, Zorraquino et al. (2008) indicated antimicrobial activity losses in beta-lactam antibiotics ranging from 9 to 35% in milk samples treated at 83 ºC for 10 minutes. On the other hand, although related studies are non-existent, the effect of the milk fat removal on

the detection of antibiotic substances could be estimated as very minimal since most antibiotics are salts present in the water phase of milk

Therefore, is important to deepen the study the effect of different milk pre-treatments on positive samples. These treatments could be included as a routine in the standard operating procedures of the monitoring laboratories in order to reduce the number of false positive results in milk residue monitoring programmes, and thus avoiding a problem for goats' milk producers and dairy industries.

4. CONCLUSIONS

In general, most of the commercial microbial inhibitor tests used to detect antibiotics are suitable for the analysis of goats' milk. The specificity of the tests improved if appropriate equipment as instrumental readers were used for the interpretation of the test results compared to the results obtained by visual reading, since for goats' milk usually intermediate colors of the test medium at the end of the incubation are obtained. In this way the testing of residue-free goats' milk samples could result in a penalty contributed to the milk producer.

The most effective milk pre-treatment for microbial inhibitor tests to reduce the number of false positive results was the fat removal followed by a heat treatment. The establishment of appropriate operational procedures in the control of the presence of antibiotics in raw goats' milk is crucial to avoid the problems associated with the presence of false positive results, contributing to limit the losses due to discarded milk and dairy products or additional confirmatory analysis costs.

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371 **Table 1.** Specificity (false positive rate) of different microbial inhibitor tests for the

372 detection of antibiotics in goats' milk (n=199)

Microbial Tests	Instrumental		Visual			p-Value
	P[b]	Specificity (%)[c]	P[b]	Q[d]	Specificity (%)[c]	MNT[a]
BR-AS Special	44	77.9	53	15	65.8	< 0.001
BRT MRL	5	97.5	16	2	91.0	0.001
CMT-Copan Milk Test	1	99.5	2	1	98.5	0.157
Charm CowSide II[e]	-	-	43	10	73.4	-
Eclipse 100	2	99.0	16	5	89.4	< 0.001
Eclipse 3G	4	98.0	5	1	97.0	1.000
Charm Blue Yellow II	11	94.5	24	10	82.9	< 0.001
Delvotest T	1	99.5	2	1	98.5	0.157
Delvotest SP-NT	12	93.9	11	2	93.4	0.317

373 [a] MNT= McNemar test

374 [b] P= positive results

375 [c] Specificity (%)= negatives/total x 100

376 [d] Q= questionable results

377 [e] Charm CowSide II= only visual results

Table 2. Classification of goats' milk samples (n=199) in 4 categories based microbial inhibitor test results (instrumental reading)

Microbial Test	Border values for classification (instrumental reading)					Number of samples per category (instrumental reading)				Number of samples per category (visual reading)			
						Negative		Positive		Negative		Positive	
	SD[1]	3 SD[1]	cut-off -3×SD[1]	cut-off	cut-off +3×SD[1]	1 (-/-)	2 (-/+)	3 (+/-)	4 (+/+)	1 (-/-)	2 (-/+)	3 (+/-)	4 (+/+)
BR-AS Special	0.7639	2.292	-5.292	-3	-0.708	114	41	26	18	107	24	15	53
BRT MRL[2]	4.461	13.38	26.617	40	53.383	191	3	4	1	177	4	2	16
CMT-Copan Milk Test	0.1521	0.456	4.044	4.5	4.956	196	2	-	1	196	-	1	2
Charm CowSide II[3]	-	-	-	-	-	-	-	-	-	135	11	10	43
Eclipse 100	0.026	0.078	0.482 0.504 0.547 0.506 0.567	0.560[4] 0.582[5] 0.625[6] 0.584[7] 0.645[8]	0.638 0.660 0.703 0.662 0.723	193	4	1	1	173	5	5	16
Eclipse 3G	0.012	0.036	0.453 0.508 0.520 0.480 0.489	0.489[9] 0.544[10] 0.556[11] 0.516[12] 0.525[13]	0.525 0.580 0.592 0.552 0.561	191	4	1	3	192	1	1	5
Charm Blue Yellow II	0.4193	1.258	4.742	6	7.258	174	14	11	-	159	6	10	24
Delvotest T	0.5334	1.6	-4.600	-3	-1.400	195	3	-	1	195	1	1	2
Delvotest SP-NT	0.3382	1.015	-4.015	-3	-1.985	160	27	6	6	180	6	2	11

[1] SD: Standard Deviation; [2] Cut-off expressed in %; [3] Charm CowSide = only visual results; [4-8] different cut-off for Eclipse 100 in each test plate; [9-13] different cut-off for Eclipse 3G in each test plate

Table 3. Results in different microbial tests for blank goats' milk samples before and after special sample treatment.

Microbial Tests	n[1]	Pre-treatments							
		No treatment		Heat treatment		Fat Removal		Fat +Heat	
		N[2]	P[3]	N[2]	P[3]	N[2]	P[3]	N[2]	P[3]
BR-AS Special	44	-	44	-	44	10	34	9	35
BRT MRL	5	-	5	-	5	-	5	-	5
CMT-Copan Milk Test	1	-	1	1	-	-	1	1	-
Charm CowSide[4]	53	12	41	50	3	25	28	53	-
Eclipse 100	2	-	2	2	-	1	1	2	-
Eclipse 3G	4	2	2	4	-	2	2	4	-
Charm Blue Yellow II	11	5	6	11	-	6	5	11	-
Delvotest T	1	-	1	-	1	-	1	-	1
Delvotest SP-NT	12	3	9	5	7	7	5	8	4

[1] n: number of positive samples, first day

[2] N: Negative result, second day

[3] P: Positive result, second day

[4] Charm CowSide = only visual results