

ROSA (RAPID ONE STEP ASSAY) FOR ANTIBIOTICS IN HONEY

David R. Legg*, Annie Baumgartner, Robert Salter and Andrew Wheeler

Charm Sciences Inc., 659 Andover St Lawrence, MA 01843 USA ,
Fax: 978-687-9216, Tel: 978-687-6200, e-mail: drl@charm.com

Abstract

ROSA is a lateral flow based strip test for the detection of antibiotics that is easily adapted to field/farm testing environments. The test uses minimal equipment (incubator) and can be read visually or with a reader. In the dairy industry, ROSA has gained rapid appeal because testing is simple, inexpensive, and can be used on farms and on transport trucks. This provides the milk industry early detection of antibiotics with the least loss of product. The ROSA tests for milk were modified as follows: Honey is diluted 1 part to 3 parts dilution buffer. The diluted sample is pipetted onto a test strip in an incubator and after 8 minutes the test is read. Developed tests have a control line and a test line. If the test line is darker than or the same intensity as the control line the sample is negative. If the test line is lighter than the control line then the sample is positive. Reader results are numerical. Negative readings are interpreted as negative and positive readings are interpreted as positive for the presence of antibiotics. Results of fortified and market samples analyzed with the ROSA Sulfamethazine Test and the ROSA Tetracycline Test are presented. The 90% positive with 95% confidence detection levels are 15.7 ppb sulfamethazine in honey and 32.9 ppb tetracycline in honey. The ROSA Test for beta-lactams detects beta-lactam drugs including cephalosporins. A lateral flow test for chloramphenicol is being developed.

Keywords: Honey/antibiotic/sulfamethazine/beta-lactam/tetracycline

Introduction

With the discovery of honeys contaminated with illegal antibiotics this year [1] [2], the honey producing industry and the regulatory agencies are interested in rapid, simple, and accurate tests for antimicrobial drugs in honey. The ROSA (Rapid One Step Assay) line of diagnostic tests, currently used worldwide by the dairy industry to detect antibiotics in milk, have been adapted for use in detecting antibiotics in honey. ROSA Tests have been evaluated and approved for use in raw milk by various regulatory agencies and independent laboratory studies [3], [4], [5]. For honey, the sample is diluted and run in the test strip in a small incubator. Within minutes, the test strip gives visual results that may also be analyzed with a reader that gives numerical results and interpretation of these results. The ROSA Tests use minimal equipment (pipette, incubator and optional Reader) and give results in less than 10 minutes.

The ROSA Sulfamethazine Test is specific for sulfamethazine (sulfadimidine). Using the ROSA Sulfamethazine Test, honey is diluted 1 part with 3 parts dilution buffer (Honey Dilution Buffer) and added to the test strip in a small test strip incubator. After 8 minutes, the test strip is removed and the two lines formed on the test strip are compared. The intensity of the color of the T (Test) line is compared to the C (Control)

line. The C line is a built-in control line to verify sample flow and proper operation. If the T line is darker than or the same intensity as the C line, the honey sample is negative. If the T line is lighter than the C line, the sample is positive for sulfamethazine. Alternatively, the test strip may be read using an optical reflectance meter called the ROSA Reader. The ROSA Reader gives numerical results and a negative or positive interpretation where 0 and negative numbers are interpreted as negative and positive numbers are interpreted as positive for the presence of sulfamethazine.

Various honey samples were tested using the ROSA Sulfamethazine Test. A standard curve and concentration response curve of a sulfamethazine-free honey fortified with sulfamethazine showing the sensitivity (90% positive with 95% confidence) to be about 16 ppb (parts per billion or ug/kg) in honey is presented. The results of several different sources of various honeys fortified at 20 ppb are also presented. A solid granular honey diluted with water may be used as a negative control diluted honey. A freeze-dried positive control standard containing sulfamethazine reconstituted with water and spiked into negative honey was used to generate positive samples.

There is no known cross-reactivity with drugs outside the antimicrobial drug family and there is no known interference from bacteria at up to 3,000,000 cfu/g honey.

The ROSA Tetracycline Test was used to test honey for antimicrobial drugs in the tetracycline family. Honey is diluted 1 part to 3 part with Buffer M, a reconstituted milk powder. A standard curve and concentration response curve of a honey fortified with tetracycline showing the sensitivity (90% positive with 95% confidence) to be about 35 ppb in honey is presented. This test has sensitivity to other tetracycline drugs including oxytetracycline and chlortetracycline at 200 ppb and 400 ppb, respectively.

The SL Beta-lactam Test detects penicillin G at 20 ppb and other beta-lactams/cephalosporins in the 20 – 200 ppb range in honey. The SL Beta-lactam Test for Honey uses the same dilution (1 part honey to 3 parts Honey Dilution Buffer) as the ROSA Sulfamethazine Test. Although these ROSA Tests are not as sensitive as some other screening tests available, such as the Charm II Tests, the ease-of-use, simple equipment, low cost and visual interpretation make the ROSA Tests applicable to the honey producing field/farm environment [6].

A ROSA test for chloramphenicol in honey is being developed.

Materials and Methods

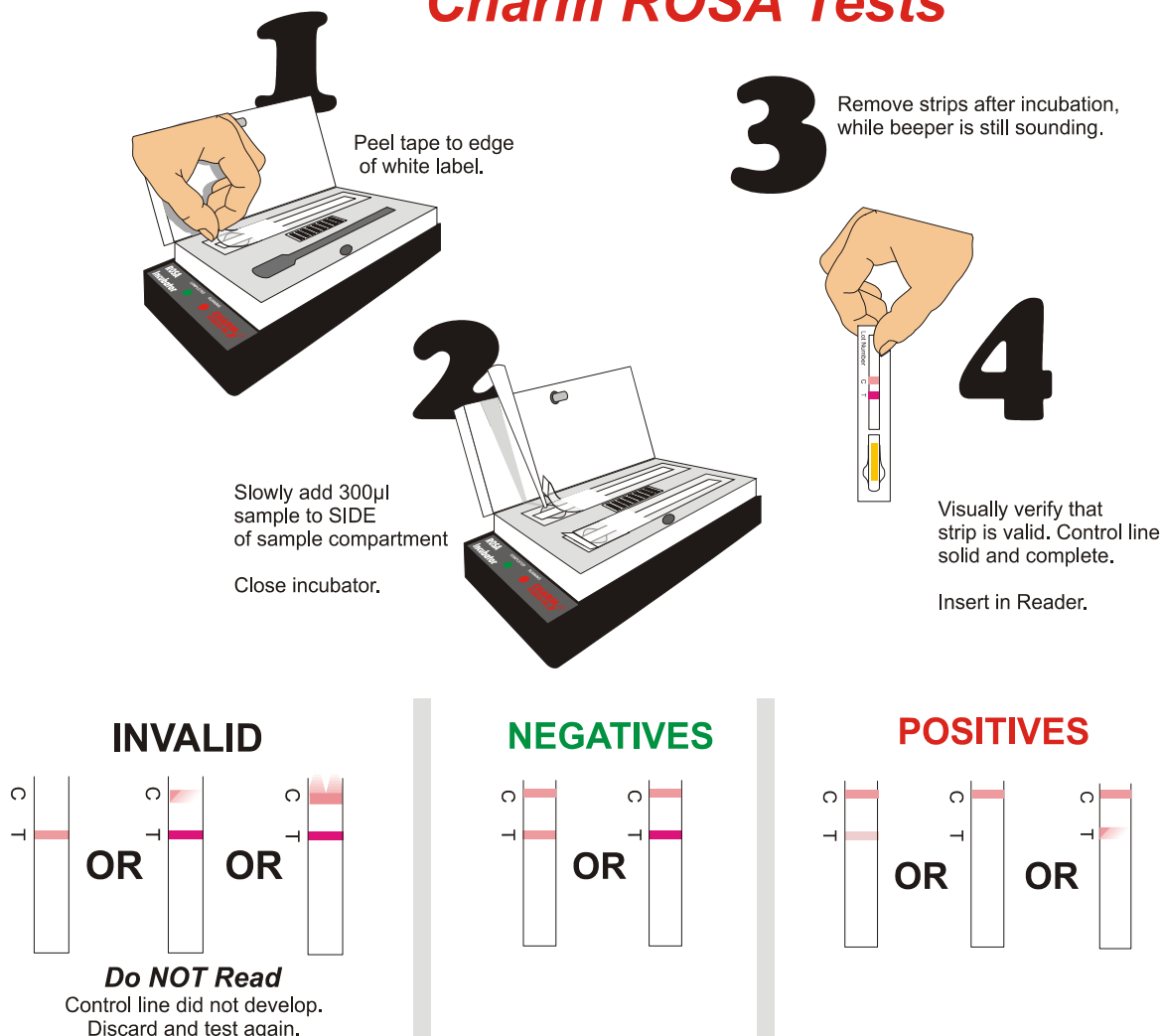
1. Reagents
 - a. ROSA test strips, individually packaged (1 test strip per test);
 - b. Honey Dilution Buffer (proprietary, liquid);
 - c. Buffer M (antimicrobial drug free milk powder reconstituted with water);
 - d. Honey Negative Control (dry granular honey reconstituted with Buffer M or Honey Dilution Buffer); and
 - e. Positive control standards (stock solutions made from USP Reference Standards [4] or freeze-dried preparations of USP Standards reconstituted)
 - f. Stored at 0 –7 °C.
2. Apparatus
 - a. Strip Test Incubator - Manufactured by Charm Sciences Inc.; operated at 56°C

- $\pm 1^{\circ}\text{C}$ (temperature verified by heat strip or thermometer) with 8 minute timing beeper and/or light available for 2 or 4 test strips
 - b. 300 μl \pm 15 μl fixed volume pipette, with tips
 - c. Balance, sensitive to 0.1 g
 - d. 1 - 5 ml adjustable volume pipette, with tips
 - e. ROSA Reader, optional – Manufactured by Charm Sciences Inc.; models ROSA Reader or Luminator Imager, or equivalent)
 - f. Sample tubes for honey dilution
3. Procedure
- a. Check that incubator temperature is $56 \pm 1^{\circ}\text{C}$ (55°C temperature strip indicator will be green) and that incubator is on a level surface. Dilute honey with Honey Dilution Buffer (for Sulfamethazine and Beta-lactams) or Buffer M (for Tetracyclines), 1 part honey to 3 parts dilution buffer (for example, 1.0 g honey and 3.0 ml dilution buffer). Mix well.
 - b. Write sample ID on label on test strip. Place a test strip in incubator flat side facing up. Re-shape any dented sample compartments to allow test strip to fit into incubator. While holding strip flat, peel back tape of ROSA test strip to indicator line, exposing sample compartment well and pad.
 - c. Pipette $300 \pm 15 \mu\text{l}$ diluted honey sample into either side of the sample compartment well. Pipette slowly so that sample does not overflow the well. Reseal tape over sample pad. Repeat steps a to c for up to 4 samples. Close cover on incubator and tighten latch.
 - d. Incubate for 8 minutes and not more than 10 minutes. Incubator will beep, or solid red LED light will switch to a flashing yellow LED light from 8 minutes until 10 minutes after closing the lid.
 - e. Remove strips from incubator. Hold strip vertically so sample compartment is down. Avoid squeezing sample compartment. Visually verify that the C line is solid and complete.
4. Determination of Results
- a. Visual Determination. Inspect if the C line is completely developed, see figure 1 step 4. If the C line is only partially developed, the test is invalid and should be discarded; this sample will need to be retested. If C line is completely developed, the test strip result is valid. A visual result is obtained by comparing the darkness of the T line to the C line. If the T line appears darker than or the same as the C line, the sample is negative. If the T line appears lighter than the C line, the sample is presumptive positive. If the T line is only partially developed, the sample is presumptive positive.
 - b. Reader Determination (optional). Insert the visually valid test strip into the ROSA Reader or Imager. Choose appropriate channel, and then enter sample ID and operator ID if necessary. Press “ENTER”. Reading and interpretation appears in 5 seconds. Negative readings and zero indicate the T line is darker than the C line and the result is interpreted as negative. Positive readings indicate the T line is lighter than the C line and the result is interpreted as positive.
5. Quality Control.
- Negative and positive controls are run on a daily basis and when presumptive positive samples are retested. Negative control strips are visually negative, with the T line clearly darker than the C line, and typically give readings less (more negative) than -400. Positive control strips are visually positive, with the T line clearly lighter than the C line, and typically give readings greater than 400.

Calibration strips are supplied with the ROSA Reader and must read daily within 20% of their printed average.

Figure 1. ROSA (Rapid One Step Assay) technology uses a lateral flow test strip and specific receptors to detect antibiotics in various samples.

Charm ROSA Tests



- 1 – Test strip is place in the incubator and the plastic tape is lifted.
- 2 – Diluted honey sample (300 ul) is slowly added to the test strip, the plastic tape is resealed, and the incubator lid is closed starting the timer.
- 3 – After 8 minutes, the test strip is removed.
- 4 – Line development is inspected to confirm solid even C line. Valid test strips may be read visually or by a Reader to determine negative or positive.

Results and Discussion

Various honeys including clover, non-clover, raw, heat-treated, early and late season honeys were run on the ROSA Sulfamethazine Test and results are presented in Table 1. Eight replicates of ten honeys were tested and gave a range of –1672 to –518 with averages from –1245 to –619. All tests were visually negative.

Table I. ROSA Sulfamethazine Test for Honey, Negative Honeys

Honey	1	2	3	4	5	6	7	8	9	10
	-1065	-901	-936	-688	-1171	-875	-766	-585	-820	-1032
	-1134	-961	-1011	-1087	-1107	-1169	-596	-617	-878	-1262
	-989	-518	-931	-1180	-1044	-930	-643	-629	-687	-1672
	-969	-819	-988	-1267	-1344	-667	-701	-532	-921	-1157
	-1004	-953	-1051	-1211	-936	-1149	-707	-584	-1059	-1091
	-970	-871	-1035	-900	-919	-669	-728	-737	-1118	-1421
	-1036	-1166	-1034	-713	-920	-873	-784	-603	-938	-933
	-989	-969	-1117	-1111	-1072	-735	-815	-664	-906	-1395
Average	-1020	-895	-1013	-1020	-1064	-883	-718	-619	-916	-1245
SD	57	183	61	225	147	196	73	61	134	242

All raw honey except as noted; 1 = clover, heat-treated; 3 = heat-treated; 4 = extra light amber honey; and 10 = non-clover (canola/alfalfa), heat-treated.

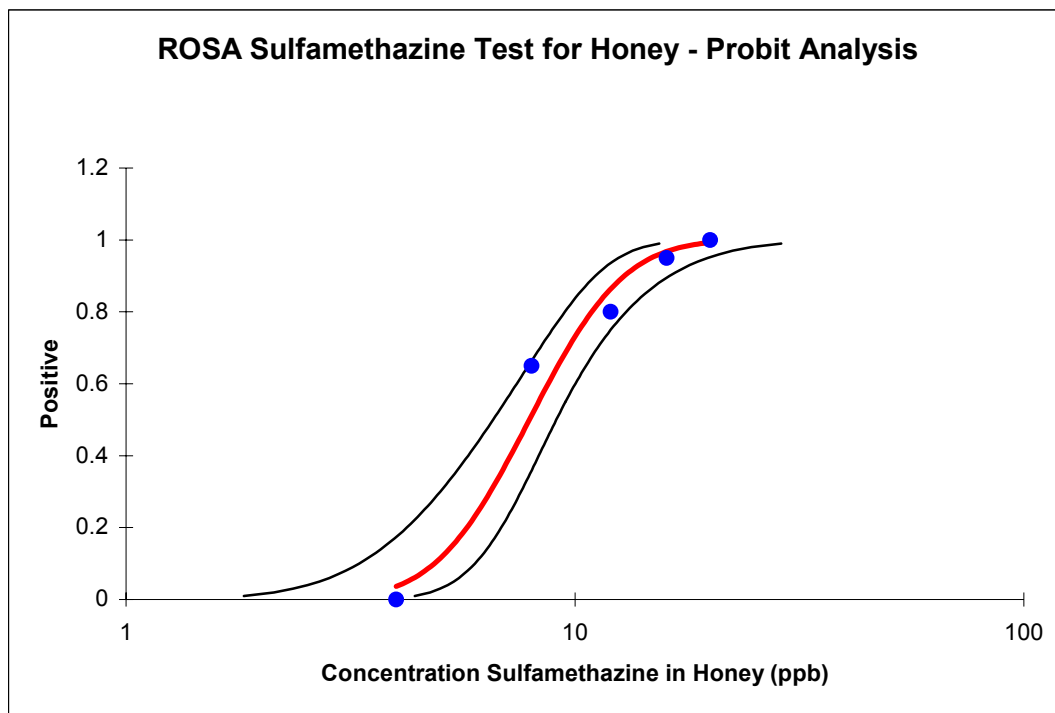
A typical negative honey with average of -1013 (honey 3) was fortified with sulfamethazine made from USP stock [7]. Twenty replicates at 0, 4, 8, 12, 16 and 20 ppb are presented in Table II to generate a standard curve and concentration-response curve. Unfortified honey gave an average of -1031 and standard deviation of 121 with all visually negative. The same honey fortified at 20 ppb gave an average of 757 and standard deviation of 170 with all visually positive.

Table II. ROSA Sulfamethazine Test for Honey, Standard Curve and Concentration-Response Curve

Sulfamethazine (ppb)	0	4	8	12	16	20
	-1293	-605	-937	-210	-97	387
	-1213	-558	-121	-147	35	588
	-1146	-534	-103	-46	450	595
	-1130	-502	-55	-38	495	611
	-1117	-425	-35	-22	509	637
	-1108	-423	-34	14	520	646
	-1099	-385	-11	43	522	701
	-1051	-367	29	72	525	724
	-1040	-340	44	104	540	726
	-1035	-333	60	369	546	728
	-1034	-323	61	379	576	736
	-1011	-321	69	436	583	740
	-988	-304	76	456	669	760
	-944	-280	77	476	693	782
	-939	-266	82	511	711	866
	-936	-189	369	526	769	877
	-934	-146	382	534	784	933
	-931	-129	454	572	818	940
	-920	-107	487	600	845	1022
	-752	-70	546	791	882	1132
Average	-1031	-330	72	271	569	757
SD	121	152	313	296	244	170
% Positive	0%	0%	65%	80%	95%	100%

Probit analysis of the concentration-response curve yielded a 90% positive concentration with 95% confidence detection level of 15.7 ppb sulfamethazine with a Pearson Chi Square of 3.3. The Probit analysis is presented in Figure 2 [8]. The 90% positive with 95% confidence level is calculated for a 1-tail confidence level from the 2-tail 95% upper confidence level provided by the Probit program in Figure 2.

Figure 2. ROSA Sulfamethazine Test for Honey, Probit Analysis and Determination of 90% Positive with 95% Confidence Level



90% Concentration =	12.88 ppb
Upper 95% Confidence =	16.25 ppb
90% with 95% Confidence = $e^{[(\ln 95\% \text{ Confidence} - \ln 90\% \text{ Conc}) * 0.84 - \ln 90\% \text{ Conc}]}$	
	e 2.75
	15.7 ppb

Para-amino benzoic acid (PABA) is a sulfa drug analog that may be found in honey. Table III shows that there is no interference from PABA at up to 1000 ppb in honey. The percent change for 1000 ppb PABA from negative honey is a 1.3 %.

Table III. ROSA Sulfamethazine Test for Honey, No Interference from PABA at 1000 ppb

	Negative Honey	1000 ppb PABA
	-1407	-1242
	-1456	-1023
	-1616	-976
	-1374	-1235
	-1157	-1140
	-1263	-1321
	-1204	-896
	-1194	-1009
	-1079	-1096
	-1121	-1255
Average	-1287	-1119
SD	171	142

The ROSA Tetracycline Test was adapted for honey using a reconstituted antimicrobial drug free milk powder as the dilution buffer. As with the ROSA Sulfamethazine Test, the honey sample is diluted one part to 3 parts dilution buffer and then run on the ROSA test. Results of six replicates of the same 10 different honeys presented for the ROSA Sulfamethazine Test in table I are presented in table IV. Results ranged from -3319 to -1394 with averages from -2684 to -1711. All tests were visually negative.

Table IV. ROSA Tetracycline Test for Honey, Negative Honeys

Honey	1	2	3	4	5	6	7	8	9	10
	-2495	-2004	-2108	-2111	-2270	-2264	-1644	-1972	-2385	-2300
	-2656	-2735	-1790	-2802	-2881	-1985	-2454	-1632	-2384	-2229
	-2908	-3319	-2136	-2842	-2318	-1841	-1668	-1394	-2234	-2938
	-2917	-2549	-2423	-2260	-2859	-1725	-1590	-1634	-3169	-2832
	-2443	-2417	-2733	-2114	-2211	-2516	-2261	-1922	-3171	-2328
Average	-2684	-2605	-2238	-2426	-2508	-2066	-1923	-1711	-2669	-2525
SD	223	481	356	367	333	322	403	237	462	332

All raw honey except as noted; 1 = clover, heat-treated; 3 = heat-treated; 4 = extra light amber honey; and 10 = non-clover (canola/alfalfa), heat-treated.

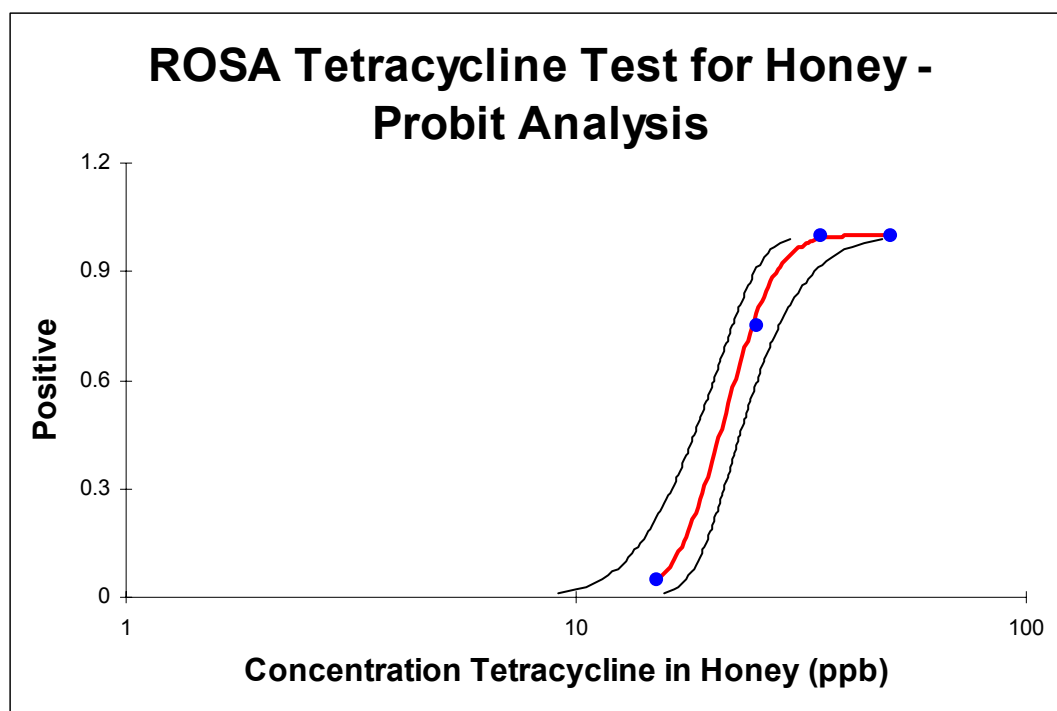
A typical negative honey was fortified with tetracycline made from USP stock [7]. Twenty replicates at 0, 15, 25, 35, and 50 ppb are presented in Table V to generate a standard curve and concentration-response curve. Unfortified honey gave an average of -1832 and standard deviation of 184 with all visually negative. The same honey fortified at 35 ppb gave an average of 525 and standard deviation of 183 with all visually positive.

Table V. ROSA Tetracycline Test for Honey, Standard Curve and Concentration-Response Curve

Tetracycline (ppb)	0	15	25	35	50
	-1820	-767	-211	166	609
	-1947	-714	-89	222	673
	-1904	-663	-75	275	683
	-1929	-583	-54	292	758
	-1660	-579	-39	439	770
	-1951	-571	2	440	775
	-1682	-484	5	465	795
	-1700	-464	6	477	811
	-1622	-443	12	490	838
	-1470	-429	14	552	896
	-1709	-396	43	559	932
	-2131	-394	58	575	937
	-1751	-319	62	575	944
	-1742	-245	123	607	955
	-1734	-205	136	650	959
	-1686	-201	164	664	977
	-2061	-165	172	686	1013
	-2079	-106	182	780	1025
	-1942	-48	241	783	1027
	-2117	-21	249	794	1050
Average	-1835	-390	50	525	871
SD	184	221	118	183	131
% Positive	0%	0%	80%	100%	100%

Probit analysis of the concentration-response curve yielded a 90% positive concentration with 95% confidence detection level of 32.9 ppb tetracycline with a Pearson Chi Square of 0.26. The Probit analysis is presented in Figure 3 [8]. The 90% positive with 95% confidence level is calculated for a 1-tail confidence level from the 2-tail 95% upper confidence level provided by the Probit program in Figure 3.

Figure 3. ROSA Tetracycline Test for Honey, Probit Analysis and Determination of 90% Positive with 95% Confidence Level



90% Concentration =	27.91 ppb
Upper 95% Confidence =	33.90 ppb
90% with 95% Confidence =	$e[(\ln 95\% \text{ Confidence} - \ln 90\% \text{ Conc}) * 0.84 - \ln 90\% \text{ Conc}]$ e 3.49 32.9 ppb

The sensitivity of the ROSA Tetracycline Test for Honey is about 400 ppb chlortetracycline and 200 ppb oxytetracycline. Other tetracyclines are also detected.

The SL Beta-lactam Test for Milk was adapted for honey using the same dilution (1 part honey sample to 3 parts Honey Dilution Buffer) as the ROSA Sulfamethazine Test for Honey. The SL Beta-lactam Test for Milk was evaluated by the AOAC-Research Institute under the U.S. FDA, CMV (Center for Veterinary Medicine) protocol for evaluation of screening tests for beta-lactam drugs in raw milk [3]. The NCIMS (National Conference of Interstate Milk Shipments) approved the SL Beta-lactam Test for Milk for official use in 1999 [9]. The evaluation included selectivity, sensitivity, ruggedness, interferences (chemical, somatic cells, and bacterial), and incurred residue testing. For honey, the honey sample is diluted 1 part to 3 parts dilution buffer prior to running the test using the same procedure as the ROSA Sulfamethazine Test and ROSA Tetracycline Test for Honey. Results of a negative honey and the same honey fortified at 10 ppb and 20 ppb penicillin G are presented in table VI. The sensitivity, defined as at least 90% positive, is 20 ppb penicillin G with an average of 459 and standard deviation of 286. This test also detects other beta-lactam drugs and cephalosporins.

Table VI. SL Beta-lactam Test for Honey, Sensitivity to 20 ppb Penicillin G in Honey

	Negative Honey	10 ppb	20 ppb
	-1445	-713	31
	-1231	-587	33
	-1226	-536	366
	-1124	-505	403
	-1092	-294	419
	-982	-291	423
	-766	-215	585
	-720	-212	655
	-720	-92	741
	-623	-68	930
Average	-993	-351	459
SD	275	220	286

There is no known cross-reactivity with drugs outside the antimicrobial drug families and there is no known interference from bacteria at less than 3,000,000 cfu/g honey.

Summary

Honey may be tested for antibiotics using the rapid, simple ROSA Tests from Charm Sciences. After a simple dilution, the sample is tested on the ROSA test strip. Up to 4 samples per incubator may be run at the same time. After an 8-minute incubation the test results may be analyzed visually or optionally with a reader. ROSA Tests for honey are available for sulfamethazine, tetracyclines, and beta-lactams. The ROSA Sulfamethazine Test detects sulfamethazine in honey at 15.7 ppb as positive 90% of the time with 95% confidence. The ROSA Tetracycline Test detects tetracycline in honey at 32.9 ppb as positive 90% of the time with 95% confidence, and also detects other tetracycline family drugs at higher levels. The SL Beta-lactam Test detects beta-lactam drugs and cephalosporins in honey, including penicillin G at 20 ppb. A ROSA test for chloramphenicol is currently in development.

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Figures and Tables

Figure 1. ROSA (Rapid One Step Assay) technology uses a lateral flow test strip and specific receptors to detect antibiotics in various samples.

Table I. ROSA Sulfamethazine Test for Honey, Negative Honeys

Table II. ROSA Sulfamethazine Test for Honey, Standard Curve and Concentration-Response Curve

Figure 2. ROSA Sulfamethazine Test for Honey, Probit Analysis and Determination of 90% Positive with 95% Confidence Level

Table III. ROSA Sulfamethazine Test for Honey, No Interference from PABA at 1000 ppb

Table IV. ROSA Tetracycline Test for Honey, Negative Honeys

Table V. ROSA Tetracycline Test for Honey, Standard Curve and Concentration-Response Curve

Figure 3. ROSA Tetracycline Test for Honey, Probit Analysis and Determination of 90% Positive with 95% Confidence Level

Table VI. SL Beta-lactam Test for Honey, Sensitivity to 20 ppb Penicillin G in Honey