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# Antiviral Substances in Raw Bovine Milk Active Against Bovine Rotavirus and Coronavirus

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ABSTRACT

After experimental contamination of bovine raw and heat-treated milks with bovine rotavirus and coronavirus strains, we observed a strong viral inhibition only with raw milks, from which virus recovery was $5 \times 10^{-4}$%. Between 30% and 80% of the virus was recovered from the heat-treated milks, depending on the level of inoculation. The antiviral substance is heat-labile (destroyed within 30 min at 100°C), precipitated by ammonium sulfate and filtrable (0.45 μm Millipore membrane). It also has neutralizing activity on tissue culture.

Bovine milks and dairy products, such as yogurts and cheeses, are important human foods in France and throughout the European Economic Community *(7)*. It is now well established that swine and bovine colostrum, raw and pasteurized milks contain, in addition to such antibacterial substances as immunoglobulins *(18)* and non-immune components - lactoperoxidase, lysozyme, lactoferrin, xanthineoxidase *(5,20)*, - also antiviral substances *(1,3,14,16)*, - inhibitors for simian rotavirus SA 11 *(6,11)*, human rotavirus (29), and calf diarrhea coronavirus *(17)*. Heat-treated milk (sterilized or UHT) do not show any rotavirus antibody activity. Human milk has the same properties. Breast feeding prevents rotavirus infections in newborns *(4,14)*. Immunoglobulin concentrates from cow's milk are prepared for human needs (8). These factors are important for resistance against infection *(14,27)*; but, on the other hand, the amount of antibodies can influence the rotavirus vaccine "take" *(26,29)*.

While studying survival of bovine rotavirus and coronavirus in milks under different conditions of temperature and environmental factors, we observed low recovery of viruses on tissue culture, only with bovine raw milk (paper under preparation). All heat-treated milks permit recovery of 50 to 90% of the viral input. We know that bovine and human rotavirus strains share common antigens *(24)*; we tried to elucidate the observed antiviral activity, considering it could be important, from a public health point of view, to anticipate the viral hazard in bovine (perhaps also in raw milk from sheep and goats) raw milks and dairy products prepared with such material, particularly soft cheeses.

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## MATERIALS AND METHODS

### *Cells*

*MA 104 cells* (Rhesus monkey kidneys) were supplied by J. Laporte, INRA, Thiverval-Grignon. Cultures were made in 75-$cm^2$ or 150-$cm^2$ plastic flasks. The growth medium was Eagle's minimum essential medium, EMEM, containing 10% fetal calf serum (FCS), 0.22 g of sodium bicarbonate/L, 0.29 g of glutamine/L, 100 U penicillin/ml, 100 μg of streptomycin/ml, buffered with 20 m*M* HEPES (N - 2 hydroxyethyl-piperazine - N' - 2 - ethanesulfonic acid). The maintenance medium was EMEM containing 2% FCS. Monolayers were harvested with a mixture of trypsin (0.25%) and versene (0.5%).

*HRT 18 cells* (Human rectal tumor) were supplied by J. Laporte *(10)*. Cultures were made in 75-$cm^2$ or 150-$cm^2$ plastic flasks. The growth medium was RPMI 1640, containing 15% FCS, 0.22 g of sodium bicarbonate/L, 0.29 g of glutamine/L, 5 mg of tylosine/L, 180 mg of lincocyne/L, buffered with 20 m*M* HEPES. The maintenance medium was RPMI 1640 supplemented by 2% FCS. Monolayers were harvested with a mixture of trypsin (0.25%) and versene (0.5%).

### *Preparation of virus stocks*

*Bovine rotavirus*. Strain RF 45, was kindly supplied by J. Laporte. Briefly, the MA 104 monolayers were washed with MEM and incubated for 3 h at 37°C. The rotavirus strain was inoculated at a low multiplicity of infection (MOI): 0.1 plaque-forming unit/cell (PFU); after 1 h of adsorption, MEM containing 10 μg of trypsin/ml, and 0.16% tryptose phosphate broth was added. After complete cytopathogenic effect (CPE), cells were disrupted by three cycles of freezing-thawing. The viral suspension was centrifuged, then the supernatant liquid was filtered through a 0.22-μm Millipore membrane. The virus stock was stored at -20°C, in 1-ml tubes. The final titer was estimated at $4 \times 10^9$ PFU/ml.

*Bovine coronavirus*. Strain G 110, was kindly supplied by J. Laporte. The HRT 18 monolayers were washed with RPMI. The coronavirus strain was inoculated at MOI: 0.1 PFU/cell. After 1 h of adsorption, the maintenance medium was added.

After complete CPE, cells were disrupted by one cycle of freezing-thawing. The viral suspension was centrifuged, then the supernatant liquid was filtered through a 0.22-μm Millipore membrane. The virus stock was stored at -20°C, in 1-ml tubes. The final titer was estimated at $2 \times 10^9$ PFU/ml.

*Milks.* Whole raw milks were obtained from a private farm, a university farm and a dairy firm. Heat-treated milk, sterilized, UHT, and pasteurized milks, were bought at a supermarket. Microfiltration or ultrafiltration fractions, permeate, retentate, and original wheys, were supplied by the Laboratory of Dairy Technology, INRA, Rennes *(15)*. Wheys from milks were extracted either by acid precipitation (pH 4.6) or rennet coagulation (pH 6.3). Heat treatments were made in a water-bath, for 30 min at 63°C, 20 sec at 72°C and 30 min at 100°C, and in an autoclave for 30 min at 120°C.

*Ammonium sulfate precipitation of wheys.* Twenty ml of acid wheys were treated with ⅔ volume of saturated ammonium sulfate solution. After centrifugation, the pellet was suspended in Phosphate Buffer Solution (PBS), then dialysed overnight at 4°C in PBS. Some fractions were filtered through a 0.45-μm membrane, or heat treated 30 min at 56°C *(19)*.

*Pancreatin treatment.* Whey samples (1 ml) were incubated with 250 μg of pancreatin/ml (Sigma), grade VI for 1 h at 37°C.

*Trypsin treatment of raw milk samples.* Whole raw milk (10 ml) samples were treated with a solution of 10, 50, 100, or 500 μg of trypsin/ml (Laboratoire MARTINET, France) and incubated for 1 h at 37°C. After trypsin treatment, samples were contaminated with the bovine rotavirus strain.

*Sample contamination.* Twenty-ml samples were contaminated with high or low inputs of bovine rotavirus or coronavirus stocks, then incubated for 2 h at 4°C.

*Sample inoculations.* We made either direct inoculation on specific tissue culture, or, especially for raw milks, we proceeded to three Freon (trichloro-1, 1, 2 trifluoroethane) treatments, with glycine buffer elution, pH 8.8, and used a Polytron homogenizer (Bioblock, France). All aqueous phases were concentrated by ultracentrifugation: 2h, 200,000 × *g*. Before plaque assay, we treated 2 × 1 ml of each rotavirus sample with a solution of 10 μg of trypsin/ml for 1 h at 37°C. All rotavirus and coronavirus samples were treated with a mixture of antibiotics: $10^4$ IU of penicillin/ml, 10 mg of streptomycin/ml, 2 μg of amphotericin B/ml and 750 μg of neomycin sulfate/ml. Incubation was 1 h at 37°C for rotavirus samples and overnight at 4°C for coronavirus samples.

*Viral plaque forming unit (PFU) assay*

*Rotavirus.* The protocol has been described by L'Haridon et al. *(12)*. Confluent monolayers of MA104 cells, 4 d old, were prepared in disposable tissue culture plates (Greiner, six wells). After three washes, samples were inoculated and held for 1.5 h at 37°C in a $CO_2$ incubator. Inoculum was removed and agarose overlay added. After 3 d, an agarose plus neutral red overlay was added. Plaques were counted after 3 to 5 d.

*Coronavirus.* The protocol has been described by Laporte et al. *(9)*. Confluent monolayers of HRT 18 cells, 4 d old, were prepared in disposable tissue culture plates (Greiner, six wells). After two washes, samples were inoculated and held for 1.5 h at 37°C in a $CO_2$ incubator. Inoculum was removed and agarose overlay added. After 2 d of incubation, the overlay was poured off. One ml of 2% rat red blood cell suspension was added, and left for 30 min at 37°C. After a PBS wash, the hemadsorption plaques were counted.

*Neutralization test.* The protocol has been described for the rotavirus neutralization test *(13,25)*. Briefly, ten-fold dilutions of milk or fractions were incubated with a suspension of $10^3$ PFU of rotavirus or coronavirus/ml overnight at 4°C. Monolayers of MA104 cells or HRT18 cells, 4 d old, were rinsed and inoculated, 200 μl of the mixture/well. After 1.5 h of incubation at 37°C in a $CO_2$ incubator, monolayers were rinsed and respective overlays dispensed. Further steps are similar to viral plaque forming unit assay. Neutralizing titer is the reciprocal of the dilution giving a 50% reduction in plaque counts.

## RESULTS

After bovine rotavirus and coronavirus contamination of raw milks and heat-treated milks, we observed a strong viral inhibition only with raw milks (Tables 1 and 2). Heat-treated milks gave between 30% and 80% of virus recovery. Microfiltration permeate and retentate, and original whey, have the same antiviral activity (Table 3). After 5 d of incubation, we recorded an absence of specific plaques or a very low recovery of infectivity after high inputs.

The same raw milk samples were Freon-treated, and viral elution made with glycine-buffer, pH 8.8 (Table 4). Recovery was 0.0005%. At the same time, all heat-treat (sterilized, UHT, or pasteurized) milks gave 100% recovery. Virus input, as inoculum, was not Freon-treated, which explains the high recovery in these milks.

TABLE 1. *Rotavirus recovery from milk samples, as a function of previous heat-treatment of the milk and of the level of virus inoculated.*

| | Inputs | | | |
|---|---|---|---|---|
| Milk samples[a] | $2.6 \times 10^4$ PFU | $1.5 \times 10^3$ PFU | $1.4 \times 10^2$ PFU | 28 PFU |
| Whole raw milk (University farm) | 0 | 0 | 0 | 0 |
| Whole pasteurized milk | $1.3 \times 10^4$[b] | $6.4 \times 10^2$ | 48 | 4 |
| | 50%[c] | 42% | 34% | 14% |
| Whole sterilized milk | $1.0 \times 10^4$ | $6.4 \times 10^2$ | 112 | 12 |
| | 38% | 42% | 80% | 42% |
| Whole UHT milk | $1.5 \times 10^4$ | $4.8 \times 10^2$ | 68 | 8 |
| | 57% | 32% | 50% | 33% |

[a]Direct inoculation on MA 104.
[b]Virus recovery PFU/sample.
[c]Percentage of virus recovery.

TABLE 2. *Coronavirus recovery from milk samples, as a function of previous heat treatment of the milk and of the level of virus inoculated.*

| Milk samples[a] | Inputs | | |
|---|---|---|---|
| | $3.3 \times 10^6$ PFU | $3.3 \times 10^4$ PFU | $3 \times 10^2$ PFU |
| Whole raw milks (University farm) | 0 | 0 | 0 |
| Whole pasteurized milk | $2.7 \times 10^{6b}$ | $1.9 \times 10^4$ | 148 |
| | 82%[c] | 56% | 44% |
| Whole UHT milk | $2.1 \times 10^6$ | $1.2 \times 10^4$ | 123 |
| | 63% | 36% | 37% |
| Whole sterilized milk | $2.6 \times 10^6$ | $2 \times 10^4$ | 222 |
| | 78% | 59% | 67% |

[a]Direct inoculation of HRT 18.
[b]Virus recovery, PFU/sample.
[c]Percentage of virus recovery.

TABLE 3. *Rotavirus recovery from acid whey and its microfiltration permeate and retentate, at different levels of inoculated virus.*

| Samples[a] | Inputs | | |
|---|---|---|---|
| | $1.3 \times 10^9$ PFU | $8.8 \times 10^6$ PFU | $8 \times 10^4$ PFU |
| Acid whey before UF pH 4.58 | $1.2 \times 10^{3b}$ | 0 | 0 |
| | 0.0001%[c] | | |
| Permeate (2 h) pH 6.62 | $7.2 \times 10^3$ | 80 | 0 |
| | 0.0006% | | |
| Retentate (2 h) pH 6.68 | $1.2 \times 10^2$ | 0 | 0 |
| | 0.00001% | | |

[a]Ultracentrifugation 2 h, $200{,}000 \times g$. Direct inoculation of pellets on MA 104.
[b]Virus recovery, PFU/sample.
[c]Percentage of virus recovery.

TABLE 4. *Rotavirus recovery from milk samples using a precipitation-extraction method.*

| Milk samples[a] | Virus recovery | |
|---|---|---|
| | (PFU/sample) | (%)[b] |
| Whole raw milk (University farm) | $2.4 \times 10^2$ | 0.0005 |
| Whole raw milk (University farm) + sterilization | $6.8 \times 10^7$ | 100 |
| Whole sterilized milk | $6.5 \times 10^7$ | 100 |
| Whole pasteurized milk | $8.6 \times 10^7$ | 100 |
| Whole UHT milk | $9.4 \times 10^7$ | 100 |

[a]Acid precipitation; Freon extraction. Inoculation on MA 104.
[b]Virus input: $5 \times 10^7$ PFU.

TABLE 5. *Recovery of rotavirus from whole raw milk (University farm) as a function of previous treatment of the milk with trypsin.*

| Trypsin treatment (μg/ml) | Virus recovery[a] | |
|---|---|---|
| | (PFU/sample) | %[b] |
| 0 | $1.6 \times 10^2$ | 0.001 |
| 10 | $1.6 \times 10^2$ | 0.001 |
| 50 | $8 \times 10^2$ | 0.005 |
| 100 | $8 \times 10^2$ | 0.005 |
| 500 | $1.4 \times 10^3$ | 0.01 |

[a]Direct inoculation on MA 104.
[b]Virus input: $1.3 \times 10^7$ PFU.

Raw milk (university farm) was treated with increasing doses of trypsin before rotavirus contamination (Table 5). With 500 μg of trypsin/ml, recovery of virus increased from 0.001% to 0.01%, which is one log more of viral infectivity. We prepared acid wheys from raw and heat-treated milks (Table 6). After heat-treatment (30 min at 63°C, 20 sec at 72°C, or 15 min at 120°C) and filtration, although we recovered about 60 to 70% of the input with wheys from heat-treated milks, we observed viral inhibition with wheys from raw milks. Heat-treatment, such as low temperature pasteurization of acid wheys from raw milks, permitted recovery of a small amount of infectivity: 2.7%. Heat-treatment of these wheys at 120°C for 15 min suppressed the inhibition and permitted the same virus recovery as with wheys from pasteurized (63%), UHT (59%), or sterilized (59%) milk. These results indicate the presence of thermolabile substances, incompletely destroyed after pasteurization (30 min at 63°C), but completely inactivated after sterilization (15 min at 120°C).

Acid and rennet wheys were prepared from two raw milks (Table 7). After dialysis, heat-treatment (30 min at 100°C), pancreatin treatment, or ammonium sulfate

TABLE 6. *Recovery of rotavirus from acid wheys heat-treated before virus inoculation.*

| Acid wheys[a] from | Treatments | | | |
|---|---|---|---|---|
| | None | 63°C, 30 min | 72°C, 20 sec | 120°C, 15 min |
| UF, permeate pH 6.6 | 0 | 0 | 0 | 59%[b] |
| Raw milk (University farm) pH 4.6 | 0 | 2.7% | 0 | 68% |
| Raw milk (University farm) + filtration, 0.45 μm pH 4.6 | 0 | 2.7% | 0 | 68% |

[a]Direct inoculation on MA 104.
[b]Percentage of virus recovery; virus input: $8.8 \times 10^5$ PFU.

TABLE 7. *Rotavirus and coronavirus recovery from previously treated wheys (acid and rennet extraction) from two raw milks.*

| Type of whey | Previous treatment | Raw milk 1 | | Raw milk 2 | |
|---|---|---|---|---|---|
| | | Rotavirus | Coronavirus | Rotavirus | Coronavirus |
| *Acid*[a] pH 4.6 | | | | | |
| | 1) control | 0 | 0 | 0 | 0 |
| | 2) dialysis (PBS) | 0 | 0 | 0 | 0 |
| | 3) 100°C, 30 min | $3 \times 10^3$[b] 39%[c] | $10^3$ 13% | $8 \times 10^3$ 100% | $7.6 \times 10^2$ 10% |
| | 4) $(NH_4)_2 SO_4$ (sat) supernatant liquid | 80 1% | 0 | $2 \times 10^2$ 2% | 0 |
| | 5) pancreatin | 0 | 0 | 0 | 0 |
| *Rennet*[a] pH 6.3 | | | | | |
| | 6) control | 0 | 0 | 0 | 0 |
| | 7) dialysis (PBS) | 0 | 0 | 0 | 0 |
| | 8) 100°C, min | $6 \times 10^3$ 79% | $3.2 \times 10^3$ 43% | $3.6 \times 10^3$ 47% | $5.5 \times 10^3$ 75% |
| | 9) $(NH_4)_2 SO_4$ (sat) supernatant liquid | $2 \times 10^2$ 2% | 0 | $8 \times 10^2$ 11% | 0 |
| | 10) pancreatin | 0 | 0 | 40 0.5% | 0 |

[a]Direct inoculation on MA 104 and HRT 18.
[b]Virus recovery PFU/sample.
[c]Percentage of virus recovery; Rotavirus input: $7.3 \times 10^3$ PFU, Coronavirus input: $7.6 \times 10^3$ PFU.

precipitation, all supernatant fractions were inoculated with rotavirus and coronavirus, respectively, $7.3 \times 10^3$ and $7.6 \times 10^3$ PFU/ml of viral input. Presence of a thermolabile substance was confirmed by heat-treatment (30 min at 100°C). Dialysis and pancreatin treatment had no effect on inhibition. Ammonium sulfate supernatant liquid shows a small amount of infectivity. This result may possibly be explained by the presence of ammonium sulfate, due to absence of dialysis. We tested the sensitivity of our viral strains to ammonium sulfate: coronavirus lost more than 2 logs of infectivity after brief incubation, 15 min at room temperature. The rotavirus strain lost ⅓ of its infectivity under the same conditions. It appears that rennet whey allows better recovery of viruses than acid whey, after heat treatment: pH for the two wheys was different, which partly explains the difference (Table 7).

By neutralization tests, we studied the specific antirotavirus and anticoronavirus activity in raw milks, heat-treated milks and their corresponding ammonium sulfate-precipitated fractions, and in ultrafiltration fractions (Table 8). Ultrafiltration retentate also has a neutralizing activity which disappears after heat-treatment (100°C for 30 min). Raw milks, whatever the source, have antirotavirus and anticoronavirus activity. This activity still persists after classical complement inactivation treatment (56°C for 30 min) or filtration through a 0.45-μm membrane. It is an antibody-like function.

TABLE 8. *Neutralization test: antirotavirus and anticoronavirus activity in milks, corresponding ammonium sulfate precipitated fractions, and ultrafiltration (UF) fractions (Permeate, retentate).*

| Milks | Origin | Rotavirus inhibition | Coronavirus inhibition |
|---|---|---|---|
| Whole raw milks | Private farm | 80[a] | 320 |
| | University farm | 80 | >320 |
| | Dairy farm | 160 | ND |
| Whole pasteurized milk | | 0 | 0 |
| Whole UHT milk | | 0 | 0 |
| Ammonium sulfate precipitated fractions from: | | | |
| Whole raw milks | Private farm | 40 | >320 |
| | University farm | 30 | >320 |
| | Dairy firm | 60 | ND |
| Whole pasteurized milk | | 0 | 0 |
| Whole UHT milk | | 0 | 0 |
| UF, retentate | | 320 | 480 |
| " + 100°C, 30 min | | 0 | 0 |
| UF, permeate | | 0 | 0 |

[a]Neutralizing titer is reciprocal of the dilution giving a 50% reduction in plaque counts.

## DISCUSSION

Bovine rotavirus or coronavirus contaminations have never been detected in bovine milks. Pasteurized milks serve to prepare dairy products such as yogurts and cheeses. Due to thermal treatment, any milk viral contamination can be eliminated. The situation is very different with raw milks, which still serve in France to prepare some sweet cheeses such as Camembert, Brie and Coulommiers. For our experiments, bovine raw milks were obtained from a private farm, a university farm, or a dairy firm. After experimental contamination, these milks showed a strong inhibiting activity to bovine rotavirus and coronavirus. Such activity "neutralized" $10^5$ PFU/ml and $10^6$ PFU/ml of bovine roavirus and bovine coronavirus infectivity, respectively.

Part of this antiviral activity was recovered from ammonium sulfate-precipitated fractions. These substances were filtrable through a 0.45-μm Millipore membrane, still active after heat treatment at 56°C for 30 min but lost their activity after heat treatment at 100°C for 30 min or 120°C for 15 min. Microfiltration and ultrafiltration are based on membrane pore size: 0.2 μm and 5 nm to 50 nm, respectively. Some immunoglobulins are present in permeate after microfiltration; on the other hand, all immunoglobulins are retained in the retentate after ultrafiltration.

Part of this antiviral activity may possibly be due to natural antitrypsin activity in milk, such as α1 antitrypsin or α2 macroglobulin. Raw milk treated with 500 μg of trypsin/ml before experimental rotavirus contamination still inhibited $10^4$ PFU/ml, compared to $10^5$ PFU/ml in the absence of trypsin treatment.

If specific rotavirus antibodies are present *(22)*, titers are low. Coronavirus antibody titers are much higher, indicating an intense circulation of the virus (J. Laporte, personal communication), as in Japan *(21)*.

We can anticipate the survival of animal viruses in contaminated raw milks used in preparing sweet cheeses. Such cheeses could be potential vehicles for viruses (2). In view of the present results, rotavirus and coronavirus contaminations of $10^5$ or $10^6$ PFU/ml, which are equivalent to $10^8$ and $10^9$ PFU/L, could be masked. Enteroviruses neutralized by coproantibodies can be reactivated by pancreatin treatment (D. O. Cliver, personal communication). In our tests, 250 μg of pancreatin/ml had no effect.

Negative results in tissue culture do not mean absence of infectivity. Because we have between 50 and 90% of virus recovery with contaminated heat-treated milks, using the same cells, we can exclude the hypothesis that our cell lines are not susceptible.

Additional factors specific to cheese preparation, like temperature (13°C, 33°C), rennet action, sodium chloride treatment, and maturation time, were studied (unpublished results). These factors interact with natural inhibitors present in raw milks (non immunoglobulin substances) *(20)*. Antiviral lipids are present in human milk *(28)*.

Most of food virologists have worked with human enteroviruses for which antiviral activity has never been shown in raw milks *(23)*. Antirotavirus and anticoronavirus activity in raw milks, on one hand, and environmental factors related to soft cheese preparation, on the other hand, are probably the most effective antiviral barriers for protection of consumers.

More work needs to be done to confirm or negate our preliminary results with other raw milks (sheep, goat, etc.) and see also if these antibody-like substances are active against human enteroviruses.

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